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Treatment of lipid-rich wastewater using free and immobilized bioemulsifier and hydrolytic enzymes from indigenous bacterial isolates

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Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy (PhD) in the Discipline of Microbiology; School of Life Sciences; College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus), Durban.

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As the candidate's supervisor, I have approved this thesis for submission.

Supervisor: Prof. A. O. Olaniran _____

PREFACE

The experimental work described in this thesis was carried out in the Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal (Westville campus), Durban, South Africa from August 2015 to December 2017, under the supervision of Professor A. O. Olaniran.

These studies show original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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DECLARATION 2 - MANUSCRIPTS UNDER REVIEW

Details of contributions to publications that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

Manuscript 1

Title: Indigenous *Acinetobacter* sp. isolated from lipid-rich wastewater in Durban produced thermostable glycoprotein bioemulsifiers

Journal: 3 Biotech

Authors:

Adegoke Isiaka Adetunji - Conceptualization, Experimental design, Data collection, Data analysis, Drafting and Editing of manuscript

Ademola Olufolahan Olaniran – Conceptualization, Experimental design, Manuscript editing

Manuscript 2

Title: Optimization of bioprocess parameters for enhanced protease production by *Bacillus aryabhattai* Ab15-ES using response surface methodology

Journal: Bioprocess and Biosystems Engineering

Authors:

Adegoke Isiaka Adetunji - Conceptualization, Experimental design, Data collection, Data analysis, Drafting and Editing of manuscript

Ademola Olufolahan Olaniran – Conceptualization, Experimental design, Manuscript editing

Manuscript 3

Title: Optimization of culture conditions for enhanced lipase production by an indigenous *Bacillus aryabhattai* SE3-PB using response surface methodology

Journal: Biotechnology and Biotechnological Equipment

Authors:

Adegoke Isiaka Adetunji - Conceptualization, Experimental design, Data collection, Data analysis, Drafting and Editing of manuscript

Ademola Olufolahan Olaniran – Conceptualization, Experimental design, Manuscript editing

Manuscript 4

Title: Treatment of lipid-rich wastewater using a mixture of free or immobilized bioemulsifier and hydrolytic enzymes from indigenous bacterial isolates

Journal: Desalination and Water Treatment

Authors:

Adegoke Isiaka Adetunji - Conceptualization, Experimental design, Data collection, Data analysis, Drafting and Editing of manuscript

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ABSTRACT

The production and discharge of raw and poorly treated lipid-rich wastewater increase yearly due to rapid urbanization and industrial growth. This results in severe environmental and health hazards by affecting the normal operations of ecosystems. Biological approach involving synergistic application of low cost bioemulsifier and hydrolytic enzymes is an efficient, cost-effective, sustainable and eco-friendly technology for the treatment of high strength lipid-rich wastewater. Therefore, the main objective of this study was to investigate the potential of a mixture of free or immobilized bioemulsifier and hydrolytic enzymes (protease and lipase) in the reduction of pollutants present in dairy and poultry processing wastewater. Glycoprotein bioemulsifiers and hydrolytic enzymes were produced extracellularly by submerged fermentation from indigenous *Acinetobacter* sp. and *Bacillus aryabhattai*, respectively. Optimization of bioprocess parameters, using response surface methodology, revealed a 4.4- and 7.2-fold increase in protease and lipase production, respectively. The bioemulsifier from strain AB9-ES (XB9) and strain AB33-ES (YB33) formed stable emulsions only with edible oils with highest emulsification indices of 79.6 and 67.9%, respectively obtained against sunflower oil. The emulsifying activity of the bioemulsifiers was stable over broad range of temperature (4-121 ºC), moderate salinity (1-6%) and pH (5.0-10.0). Comparative study of biochemical profiling of both free and immobilized hydrolytic enzymes showed no change in the optimum temperature and pH of both enzyme preparations for maximum activity. However, in comparison to free enzymes, the immobilized enzymes recorded enhanced stability over the investigated pH and temperature ranges. Kinetics properties revealed enhanced enzyme-substrate affinity and increased catalytic efficiency of the immobilized enzymes when compared to soluble enzymes. In addition, the immobilized enzymes were more stable when stored at 4 and 25 °C and reusable for more than five consecutive cycles. These hyper-active and highly stable bioproducts were utilized in cocktail in both soluble and entrapped form for the batch biodegradation of pollutants present in lipid-rich wastewater. Biodegradability of the wastewater was assessed by measuring the reduction of COD and lipid content at time intervals under varying incubation conditions. In dairy wastewater treated at 37 °C without pH

adjustment, maximum COD (60.51 and 65.19%) and lipid (47.98 and 63.53%) reduction efficiencies were recorded at 120 h using free and immobilized bioproducts, respectively. However, under these conditions, maximum COD (86.44 and 93.65%) and lipid (51.62 and 69.06%) removal efficiencies of poultry processing wastewater were observed at 120 h when treated with free and immobilized bioproducts, respectively. At temperature of 50 °C and pH 8.0, there was enhanced reduction of organic pollutants, with maximum COD (65.96 and 77.52%) and lipid (55.22 and 71.12%) removal efficiencies obtained in dairy wastewater at 72 h when using free and immobilized bioproducts, respectively. In the case of poultry processing wastewater, optimum COD (90.29 and 94.72%) and lipid (63 and 76.66%) removal was recorded at 72 h when treated with free and immobilized bioproducts, respectively. Reusability studies suggest that the immobilized bioproducts could be reused for up to six and seven times for the treatment of dairy and poultry processing wastewater, respectively. Findings from this study suggest the efficient, cost-effectiveness, sustainability and synergistic application of the developed immobilized bioemulsifier and hydrolytic enzymes in the removal of pollutants present in dairy and poultry processing wastewater.

This thesis is dedicated to:

My beloved parents, late (Pa) Salawudeeen & Alh (Mrs) K. A. Adetunji

for their love, support, prayers and inspiration during the course of my academic career

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INTRODUCTION

1.1 BACKGROUND

Lipid-rich wastewater is defined as a wastewater that consist of lipids along with broad spectrum of dissolved organic and/or inorganic substances in suspension at high concentrations (Adulkar and Rathod, 2015). Lipid-contaminated wastewater may be of mineral, animal, or vegetable origin from a wide variety of commercial establishments including slaughterhouses, sausage and meat product factories, restaurants, fish processing industries, dairy product industries, leather industries, edible oil refineries, wool scouring factories, petrochemical industries and metal processing industries (Chowdhury *et al*., 2010; Damasceno *et al*., 2014; Bala *et al*., 2015; Porwal *et al*., 2015; Vendramel *et al*., 2015). It is characterized by high chemical oxygen demand (COD), biochemical oxygen demand (BOD), proteins, total suspended solids (TSS), nitrogen, phosphorus, sulphates along with many other toxic compounds, depending on the operations and products from the producing industries (Farizoglu and Uzuner, 2011; Couras *et al*., 2015). Lipid-rich wastewater occurs as free- floating oil, unstable oil-water emulsions and highly stable oil-water emulsions (Awaleh and Soubaneh, 2014). The oil content is usually classified into four categories according to its physical form, *viz.* free oil, dispersed oil, emulsified oil and dissolved oil (Coca *et al*., 2011).

The production and discharge of raw and poorly treated lipid-rich wastewater increase every year due to rapid urbanization and industrial growth (Affandi *et al*., 2014). These industries consume large amounts of water for various processes, equipment and washing facilities as well as, for product production resulting in the release of huge volume of wastewater, which, if left untreated, could lead to increased disposal and severe pollution problems, thereby creating environmental hazards and hampering the normal operations of the ecosystems (Porwal *et al*., 2015).

Due to stringent regulations for effluent discharge and increasing drive for re-use of treated wastewater, treatment of lipid-rich wastewater has become an issue of great necessity (Qin *et al*., 2007). Factors such as costs, wastewater composition (high, medium, or low strength), treatment efficiency, regulatory limitations and end use of wastewater influence the choice of treatment methods for lipid-rich wastewater (Rajasulochana and Preethy, 2016). Various physicochemical techniques have been studied for their applicability in the treatment of lipid-rich wastewater (Rodrigues *et al*., 2007; Kushwaha *et al*., 2011). These mainly include flotation, sedimentation, ozonation, neutralization, chemical coagulation, gravity separation, grease trap, oil-water separator, parallel-plate coalesces, cyclone separation, granular media filtration, ultrafiltration, microfiltration among others (Chipasa, 2001). However, these techniques remain

unsatisfactory due to low treatment efficiency in the removal of dissolved and/or emulsified fats, operational difficulties, high operational costs, generation of secondary pollutants and higher quantities of solids, and use of chemical agents (Guolin *et al*., 2011). This makes it imperative for the establishment of efficient, cost-effective, eco-friendly and sustainable technologies that can serve as alternatives to existing treatment systems for lipid-rich wastewater.

Biological method is an emerging treatment technology that utilizes metabolic potential of microorganisms for the elimination of hazardous contaminants present in lipid-rich wastewater under aerobic or anaerobic conditions, or a combination of both *via* complete degradation or sequestration (Soleimaninanadegani and Manshad, 2014; Nzila *et al*., 2017). It is a method of choice nowadays in comparison to other remediation technologies due to its cost-effectiveness and wide environmental acceptability (Chanthamalee *et al*., 2013). Pollutants removal efficiency is enhanced by the presence of microorganisms with appropriate metabolic activities, adequate nutrient concentration, pH and temperature (Das and Chandran, 2011). The microbes utilize the pollutants as source of carbon, thereby converting them into innocuous products through production of appropriate metabolites such as bioemulsifier or enzymes by direct cell contact (Karigar and Rao, 2011; Franzetti *et al*., 2012).

The formation of emulsions through microbial production and release of bioemulsifier is an important process in the treatment of lipid-rich wastewater (Rahbari-Sisakht *et al*., 2017). Bioemulsifiers facilitate enzymatic activity and degradation of fats and oils by increasing their solubility and bioavailability (Daverey and Pakshirajan, 2011; Damasceno *et al*., 2012, 2014). This enhances the rate of pollutant dissolution and utilization by microorganisms (Banat *et al*., 2010).

The use of biocatalysts is a promising technology for the treatment of high fat-containing wastewater. An alternative to conventional approaches that is attracting growing interest is the use of enzymes, which significantly reduce the level of organic pollutants in the wastewater by means of enzymatic catalysis and enhance better performance of microbial community at the later stage of biological treatment process (Cammarota and Freire, 2006; Alexandre *et al*., 2011; Valladão *et al*., 2011; Demarche *et al*., 2012). Enzymes are very versatile, efficient under mild conditions, stable under typical treatment conditions and selectively act on the target compounds in wastewater (Ferreira-Leitão *et al*., 2017).

Immobilization of the above-mentioned metabolites improves lipid-rich wastewater treatment efficiency and further enhances reclamation and re-usability of immobilized bioproducts, thus reducing overall costs (Jeganathan *et al*., 2006, 2007; Saranya *et al*., 2014; Suryanti *et al*., 2017). In addition, the support materials protect the bioproducts from harsh environmental conditions, including mechanical stress and extreme pollutant concentrations (Datta *et al*., 2013; Mohamad *et al*., 2015; Lee *et al*., 2017). This further enhances

degradation capacity of the bioproducts in comparison to free form. Immobilization can be achieved by adsorption, covalent binding, entrapment or cross-linking technique using organic and/or inorganic support materials (Saranya *et al*., 2014; Rehm *et al*., 2016).

1.1.1 GENERAL CHARACTERISTICS OF DAIRY WASTEWATER

In many parts of the world, dairy industry is generally considered as the largest type of food industry and as a major source of food processing wastewater, contributing to pollution (Britz, 2006). In South Africa, due to ever-increasing demand for milk and milk products, dairy industry represents one of the largest sources of industrial effluents generating less than 5 million m^3 of waste effluent per annum (WRC, 1989; Briao and Granhen Tavares, 2007). Since dairy industry produces different products including milk, butter, yoghurt, ice cream and cheese, the volume and composition of wastewater generated vary depending on the type of product being produced, the nature and scale of operation and the design of the plant (Liu and Haynes, 2011).

Most of the wastewater volume generated in dairy industry originates from cleaning processes; equipment used for production, milk/milk products spills etc. (Kushwaha *et al*., 2011). Milk loss to the wastewater stream can amount to 0.5-2.5% of the incoming milk and can be as high as 3-4% (Munavalli and Saler, 2009). Dairy wastewater is usually generated intermittently thus changing the flow rates of this wastewater. Wastewater generated from milk processing can be separated into two classes, *viz*. wastewater with high flow rates and effluent produced in small milk-transformation units (e.g. in cheese production) (Castillo *et al*., 2007).

Dairy wastewater is concentrated in nature and contains high amounts of organic molecules such as proteins (casein), lactose and lipids, contributing largely to high levels of BOD, COD, oil-grease content and suspended solids (SS) (Kohle *et al*., 2009; Farizoglu and Uzuner, 2011; Adulkar and Rathod, 2015). In addition, it is composed of whey, cream, separator and clarifier waters, yoghurt starter culture, detergents, inorganic salts, sanitizers and stabilizing compounds (Singh *et al*., 2014). Dairy wastewater appears white in color with heavy black sludge and strong butyric acid odor due to the decomposition of casein (Shete and Shinkar, 2013). It is slightly alkaline in nature and becomes acidic quite rapidly upon fermentation of milk sugar to lactic acid (Pathak *et al*., 2016). In industrial dairy wastewater, nitrogen originates mainly from milk proteins in form of either organic or inorganic nitrogen source. Phosphorus is found mainly as orthophosphate, polyphosphate, or in organic forms (Demirel *et al*., 2005; Kushwaha *et al*., 2011). Suspended solids in dairy wastewater originate from coagulated milk, cheese curd fines or flavoring ingredients, concentration of which varies in the range of 0.024-4.5 g/L (Kushwaha *et al*., 2011). The pH of dairy wastewater varies in the range of 4.7-11; large fluctuations in pH occur due to the use of acidic and

caustic cleaning agents (Passeggi *et al*., 2009; Liu and Haynes, 2011). Significant concentrations of selected elements including sodium, potassium, calcium, magnesium, iron, cobalt, nickel and manganese are also present in dairy wastewater (Slavov, 2017). High concentrations of sodium and chloride result from the use of large amounts of alkaline cleaners in dairy plants (Demirel *et al*., 2005).

1.1.2 GENERAL CHARACTERISTICS OF POULTRY PROCESSING WASTEWATER

The global meat production in the past few years has recorded a tremendous increase with a projection of steady doubling growth until 2050 (Mekonnen and Hoekstra, 2012; Bouwman *et al*., 2013; FAO, 2013). Poultry industry represents the largest sector of the South African (SA) agricultural sector, contributing more than 16% of its share of gross domestic product. It has evolved over more than 100 years from a set of backyard activities into a complex and highly integrated industry with increasing production of chicken. Approximately 76% of the birds in the SA poultry industry are used for meat production, while the remaining 24% are used in the egg industry (The South African Poultry Association Industry Profile, 2015). The annual consumption of poultry products in SA surpasses the overall intake of all other animal protein sources. In other words, 65.5% of locally produced animal protein consumption is supplied by the poultry industry (The South African Poultry Industry Profile, 2012).

Poultry processing industries consume considerably large amounts of water for cleaning, rinsing of carcasses and poultry products as well as for sanitizing and disinfecting facilities and equipment, depending on the type of process employed, equipment used, productivity of the processing facility and waste management practices (Bustillo-Lecompte and Mehrvar, 2015). The World Bank Group (2007) describes a slaughterhouse plant as a meat processing facility that may consume approximately 2.5-40 m^3 of water per metric tons of meat produced. A typical SA poultry processing industry uses 15-20 L of water per bird processed (CSIR, 2010). Chávez *et al*. (2005) reported that about 12-24 L of wastewater are generated per bird slaughtered, and the poultry industry shows substantial environmental impacts related to water consumption with a consequent generation of greater volume of high-strength wastewater (Hamawand *et al*., 2017).

Poultry industry wastewater is characterized by high concentrations of biodegradable organic matter, mainly lipids and proteins (Valladão *et al*., 2007). Lipids accounted for more than 67% of the particulate COD of the slaughterhouse wastewater (Damasceno *et al*., 2012). Dors *et al*. (2013) have reported that poultry industry wastewater contains high concentrations of organic matter inform of COD (39,300 mg/L), lipids (2,005 mg/L) and SS (8,390 mg/L). In addition, this wastewater is composed of considerable amounts of phosphorus, nitrogen, organic carbon, heavy metals, nutrients, detergents, disinfectants etc. (Debik and Coskun, 2009; Bustillo-Lecompte and Mehrvar, 2015). The high contents of organic matter in the poultry

processing wastewater can be attributed to residual blood, excreta, fat, feather as well as stomach and intestinal mucus in the wastewater (Debik and Coskun, 2009).

1.1.3 IMPACTS OF LIPID-RICH WASTEWATER

Lipid-rich wastewater has been considered as an industrial wastewater in the category of agricultural and food industries, and classified as one of the most hazardous wastewater to the environment by the United States Environmental Protection Agency (USEPA) (USEPA, 2004). Wastewater from dairy and poultry processing industries are rich in biodegradable organic matter and nutrients, and normally contain high levels of fats and proteins contributing to their low biodegradability coefficient (Liu and Haynes, 2011). Improper disposal of this wastewater, especially when untreated or poorly treated poses serious threats to the receiving environment by causing air pollution and contamination of surface and underground water (Shete and Shinkar, 2013). Lipids form a layer on the receiving water surface, thereby reducing the dissolved oxygen transfer rate into an aerobic process (Abd El-Gawad, 2014). High lipid contents in biological wastewater treatment plants cause a severe obstacle by interfering with cell-aqueous phase transfer rates through the formation of lipid coat around the biological floc. This further led to the development of filamentous microorganism blooms (bulking) and floating sludge with undesirable physical characteristics (Cammarota and Freire, 2006; Ferreira-Leitão *et al*., 2017). Furthermore, poor activity attributed to excessive amounts of lipids in the wastewater inhibit acetogenic-methanogenic microorganisms, resulting from accumulation of long chain fatty acids (Battimelli *et al*., 2010; Zhang *et al*., 2011). In addition, due to viscous nature of lipids, their presence in wastewater cause clogging of drain pipes and sewer lines resulting in the release of unpleasant odor, corrosion of sewer lines and unsightly appearance on the surface of receiving water bodies (Husain *et al*., 2014; He *et al*., 2015). Nutrients such as nitrogen present in lipid-rich wastewater led to eutrophication of receiving water bodies (Kushwaha *et al*., 2011). High SS contents in oily wastewater has been an issue of concern. This slows down degradation rate and results in scum layer production (Hejnfelt and Angelidaki, 2009). In the agricultural sector, release of oily sludge transforms the physicochemical properties of soil with deleterious effect on the soil morphology (Robertson *et al*., 2007). It affects the water absorption capacity of the soil, reduces its hygroscopic moisture and hydraulic conductivity (Trofimov and Rozanova, 2003; Suleimanov *et al*., 2005). Plants grown on such soil encounter inhibition in seed germination coupled with access to fewer nutrients, which eventually lead to restricted growth (Al-Mutairi *et al*., 2008).

1.2 SCOPE OF THIS STUDY

It is obvious that the discharge of untreated and inadequately treated lipid-rich wastewater upsurges every year due to increased productivity from various food processing industries, posing serious environmental

and health hazards (Affandi *et al*., 2014). These industries encounter challenges to accomplish the requirements imposed by the stringent regulations for wastewater discharge and disposal along with high costs of treatment of huge volume of wastewater (Qasim and Mane, 2013). Because of raising austere regulations by environmental authorities for the removal of organic components from the wastewater, specificity becomes very crucial (Ferreira-Leitão *et al*., 2017). Conventional effluent treatment technologies are inept to eliminate all contaminants, especially dissolved and /or emulsified fat in the wastewater. Combined use of inexpensive bioemulsifier and hydrolase represents a key development that has recently gained attention for selective removal of target pollutants in the wastewater (Damasceno *et al*., 2012, 2014; Silva *et al*., 2013; Saranya *et al*., 2014; Suryanti *et al*., 2017). Immobilization in appropriate support materials is a cost-effective strategy for improved productivity and operational stability of the bioproducts (Villegas *et al*., 2016). However, knowledge on the comparative study of free and immobilized bioproducts (bioemulsifier and hydrolytic enzymes) for lipid-rich wastewater treatment is currently lacking. It is therefore imperative to investigate the synergistic application of these bioproducts as a novel approach for the treatment of high strength lipid-rich wastewater. These bioproducts facilitate degradation of lipid-rich wastewater by triggering two simultaneous processes namely; emulsification of fat particles and enzymatic catalysis, leading to increased reduction of organic load in the wastewater and eliminate the problem associated with the discharge of wastewater.

This research provides a holistic approach for the effective removal of pollutants present in lipid-rich wastewater. It hopes to establish new knowledge that will aid in the development of methods for the treatment of lipid-rich wastewater. It will promote the drive for re-use of treated wastewater and conserve normal operations of the ecosystems. Reports obtained from the physico-chemical characterization of the wastewater provides information on the state of contamination of the wastewater, and these would be useful for relevant environmental protection agencies and future scientists. Finally, the use of immobilized bioemulsifier and hydrolytic enzymes will provide a realistic, efficient, sustainable, and cost-effective approach for the degradation of pollutants in the wastewater. Therefore, this research focused on investigating the potential of a mixture of free or immobilized bioemulsifier and hydrolytic enzymes (protease and lipase) in reduction of pollutants from dairy and poultry processing wastewater collected from selected sites in KwaZulu-Natal province of South Africa.

1.3 HYPOTHESIS

It is hypothesized that bioemulsifier and hydrolytic enzymes (protease and lipase) produced from indigenous bacterial isolates will be effective in the reduction of pollutants from dairy and poultry

processing wastewater. It is further hypothesized that the immobilized bioproducts will be more efficient and robust than free form in the degradation of organic load present in the wastewater.

1.4 AIM

1.4.1 To investigate the potential of a mixture of free or immobilized bioemulsifier and hydrolytic enzymes (protease and lipase) from indigenous bacterial isolates in reduction of pollutants present in dairy and poultry processing wastewater

1.5 SPECIFIC OBJECTIVES

1.5.1 To collect lipid-rich wastewater samples from selected sites in the KwaZulu-Natal province, South Africa

1.5.2 To isolate, screen, identify and carry out phylogenetic analysis on the selected bioemulsifier-, protease- and lipase-producing bacteria

1.5.3 To produce bioemulsifier, protease and lipase from the selected indigenous bacteria isolated from lipid-rich wastewater by submerged fermentation

1.5.4 To optimize bioprocess parameters for enhanced protease and lipase production from indigenous bacteria isolated from lipid-rich wastewater using response surface methodology

1.5.5 To immobilize protease and lipase in calcium alginate beads using entrapment method

1.5.6 To determine the effect of physicochemical parameters on emulsifying activity as well as on free and immobilized protease and lipase activity

1.5.7 To determine the percentage removal efficiency of organic load from lipid-rich wastewater using a mixture of free or immobilized bioemulsifier and hydrolytic enzymes

1.6 KEY QUESTIONS TO BE ANSWERED

1.6.1 Do indigenous bacteria isolated from lipid-rich wastewater demonstrate bioemulsifying, proteolytic and lipolytic activity?

1.6.2 How effective is response surface methodology in the optimization of fermentation parameters for enhanced protease and lipase production?

1.6.3 What are the optimum conditions for maximum bioemulsifying, protease and lipase activity?

1.6.4 How stable and reusable is immobilized bioemulsifier and hydrolytic enzymes in the treatment of lipid-rich wastewater?

1.6.5 How effective is a mixture of free or immobilized bioemulsifier and hydrolytic enzymes in the treatment of lipid-rich wastewater?

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CHAPTER TWO

A REVIEW ON PRODUCTION, PROPERTIES AND POTENTIAL APPLICATIONS OF MICROBIAL SURFACTANTS

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A review on production, properties and potential applications of microbial surfactants

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Abstract Microbial surface-active compounds are amphipathic molecules that consist of hydrophilic and hydrophobic moieties, which permit their partition at the interface between fluid phases of varying degree of polarity. They are divided into two main groups namely, bioemulsifier and biosurfactant, based on their molecular weight. These biomolecules are synthesized by diverse groups of microorganisms mainly bacteria, fungi and yeasts. Their production is significantly influenced by substrate type, fermentation technology and microbial strains. Microbial surfactants are generally categorized into different groups based on their chemical nature, ionic charge and producing organisms. Due to their inherent broad functional properties and diverse synthetic capabilities of the microbes, microbial surfactants are mostly preferred than their chemical counterparts for various industrial and biomedical applications including bioremediation, enhanced oil recovery, clean-up of oil-contaminated sites, as additives in cleaning products and laundry formulations, and as emulsion-stabilizing agents in the medical, food and cosmetic industries. This review provides an insight on the methods for detection of microbial surfactant production as well as discusses chemical nature of these surface active compounds. It further elucidates the various factors influencing biosurfactant production coupled with techniques used for recovery and purification of these biomolecules for various industrial and environmental applications.

Keywords Microbial surfactants; Bioemulsifier; Biosurfactant; Microorganisms; Production; Application

Introduction

Microbial surface-active compounds (SACs) are amphipathic and structurally diverse group of compounds consisting of both hydrophilic and hydrophobic domains which preferentially partition between liquid interfaces with varying degrees of polarity and hydrogen bridges, including oil-water or air-water interfaces (Santos et al. 2016). Usually, the hydrophobic moiety is a hydrocarbon chain consisting of saturated or

unsaturated long-chain fatty acids whereas, the hydrophilic moiety is made up of ionic (cationic or anionic), non-ionic, amphoteric, amino acids or polysaccharides (Silva et al. 2014a; Mao et al. 2015). They are categorized into two groups: low-molecular weight and high- molecular weight SACs. The low molecular weight SACs, also known as biosurfactants are recognized for robust surface activity by reducing surface and interfacial tension between different phases as well as possess low critical micelle concentration (CMC) and form stable emulsions (Batista et al. 2006; Uzoigwe et al. 2015). The ability to lower surface and interfacial tension is caused by the adsorption of the biosurfactant to different phases, resulting in more interaction and mixing of dissimilar phases, which functions to solubilize hydrophobic substrates (Satpute et al. 2010a; Walter et al. 2010; Uzoigwe et al. 2015). They composed of biochemical complexes including a wide range of molecules such as sugars, fatty acids, amino acids and functional groups such as carboxylic acids. The glycolipids comprise of different sugars linked to β-hydroxyl fatty acids while lipopeptides consist of cycloheptapeptides with amino acids bonded to fatty acids of different chain length (Uzoigwe et al. 2015).

On the contrary, bioemulsifiers are high molecular weight polymers that are more effective in forming and stabilizing oil-water or water-oil emulsions, but do not necessarily reduce surface or interfacial tension (Gudiña et al. 2015). They consist of polymeric structures including polysaccharides, proteins, lipids, and complex mixtures such as heteropolysaccharides, lipopolysaccharides, lipoproteins etc. (Perfumo et al. 2009; Smyth et al. 2010a; Sekhon-Randhawa 2014). These combinations confer upon them better emulsifying potential and ability to stabilize emulsions. Bioemulsifiers are produced by different bacteria (Rosenberg et al. 1979; Navon-Venezia et al. 1995; Ilori et al. 2005; Martínez-Checa et al. 2007; Dastgheib et al. 2008; Franzetti et al. 2008, 2012; Zheng et al. 2011, 2012), yeasts (Monteiro et al. 2009, 2010, 2012) and filamentous fungi (Silva et al. 2014b). Bioemulsifiers are classified into different groups, based on their inherent chemical structure and the identity of the microorganisms that synthesize them (Ron and Rosenberg 2001). These include polymeric and particulate surfactants (Kapadia and Yagnik 2013).

The global demands for surfactants has surpassed 2.5 million tons in 2002, was less than $1735.5 million in 2011 and projected to reach $2210.5 million in 2018, equivalent to the mean annual growth rate of less than 4% from 2011-2018 (Sekhon et al. 2012). Due to their biological source, bioemulsifiers are considered to be less toxic, easily biodegradable with greater environmental compatibility; exhibit high selectivity and specific activity at extreme pH, temperatures and NaCl concentrations. They can be produced *in situ* from renewable and readily available economical raw materials, and possess similar or better emulsifying activities in comparison to chemically-prepared conventional surfactants (Pacwa-Plociniczak et al. 2011; Franzetti et al. 2012; Vaz et al. 2012; Kapadia and Yagnik 2013; Santos et al. 2013; Banat et al. 2014).

Bioemulsifiers possess unique and distinct diverse functional properties including emulsification, wetting, foaming, solubilization, corrosion-inhibition, dispersion etc., making them a suitable candidate in various biotechnological applications such as bioremediation; as additives in cleaning and laundry formulations, and as emulsion-stabilizer in the foods, cosmetics or pharmaceutics (Amaral et al. 2006; Martínez-Checa et al. 2007; Dastgheib et al. 2008; Banat et al. 2010; Monteiro et al. 2010; Franzetti et al. 2012; Zheng et al. 2012; Colin et al. 2013).

This review therefore provides an overview of the microbial production of biosurfactants/bioemulsifiers, detailing detection methods, influencing factors and kinetics of production of these surface active compounds. It also discusses key features including chemical nature, recovery and purification techniques as well as potential industrial and biomedical applications of biosurfactants/bioemulsifiers.

Biosurfactants/bioemulsifier-producing microorganisms

In recent decades, there has been a renewed interest in bioprospecting for microorganisms with potential to produce tensioactive molecules with robust surfactant characteristics (Silva et al. 2014a). Surface-active compounds are produced by various groups of microorganisms mainly bacteria (Gram-positive and Gram-negative), fungi, and yeasts. The quantity of biosurfactant production depends on the microorganism type and source of isolation. Various microbial communities have been reported to produce biosurfactants/bioemulsifiers (Macdonald et al. 1981; Cooper and Paddock 1983; Cameron et al. 1988; Singh and Desai 1989; Zinjarde et al. 1997; Abraham et al. 1998; Kakugawa et al. 2002; Lin et al. 2003; Lukondeh et al. 2003; Rau et al. 2005; Amaral et al. 2006; Maneerat et al. 2006; Perfumo et al. 2006; Morita et al. 2007; Teichmann et al. 2007; Das et al. 2008a, 2008b; Monteiro et al. 2012; Ozdal et al. 2017). A list of some biosurfactant-producing microorganisms is shown in Table 1.

Table 1 Some biosurfactant-producing microorganisms

Microorganism	Biosurfactant	Reference
Microbacterium sp. MC3B-10	Microbactan	Camacho-Chab et al. 2013
Pseudomonas aeruginosa ATCC 9027	Rhamnolipids	Díaz De Rienzo et al. 2016
Bacillus thailandensis E264	Rhamnolipids	Díaz De Rienzo et al. 2016
Pseudomonas fluorescens	Viscosin	Banat et al. 2010
Bacillus subtilis SPB_1	Surfactin/Iturin	Mnif et al. 2016
Bacillus subtilis	Subtilisin	Sutyak et al. 2008
Bacillus subtilis JB1	Subtilisin	Sung et al. 2010
Acinetobacter calcoaceticus PTCC 1316	Emulsan	Amani and Kariminezhad 2016
Acinetobacter radioresistens KA53	Alasan	Toren et al. 2001
Acinetobacter calcoaceticus A2	Biodispersan	Rosenberg and Ron 1989
Leuconostoc mesenteroides	Viscosin	Banat et al. 2010
Serratia marcescens	Serrawettin	Matsuyama et al. 2011
Serratia rubidaea SNAU02	Rhamnolipids	Nalini and Parthasarathi 2014
Gordonia sp. BS29	Extracellular bioemulsan	Franzetti et al. 2009
Pedobacter sp. MCC-Z	Glycolipid-protein complex	Beltrani et al. 2015
Streptomyces sp. MAB36	Glycolipid	Manivasagan et al. 2014
Rhodotorula babjevae YS3	Sophorolipids	Sen et al. 2017
Halomonas eurihalina	Glycoprotein	Gutiérrez et al. 2007
Debaryomyces polymorphus	Carbohydrate-lipid complex	Nerurkar et al. 2009
Paenibacillus sp. #510	Oligosaccharide-lipid complex	Gudiña et al. 2015
Achromobacter sp. HZ01	Lipopeptide	Deng et al. 2016
Ochrobactrum pseudintermedium C1	Glycoprotein	Bhattacharya et al. 2014
Aspergillus ustus MSF3	Glycolipoprotein	Kiran et al. 2009
Penicillium chrosogenum SNP5	Lipopeptide	Gautam et al. 2014
Candida bombicola	Sophorolipids	Felse et al. 2007
Candida lipolytica UCP 0988	Rufisan	Rufino et al. 2011
Candida sphaerica UCP 0995	Lunasan	Luna et al. 2011
Yarrowia lipolytica IMUFRJ 50682	Yansan	Amaral et al. 2006
Ustilago maydis	Cellobiose lipids	Teichmann et al. 2007
Pseudozyma fusifornata	Mannosylerythritol lipids	Morita et al. 2007
Trichosporon mycotoxinivorans CLA2	Lipid-polysaccharide complex	Monteiro et al. 2012

These microorganisms are found in different habitats including water (sea, fresh water and ground water), land (soil, sediment and sludge) and harsh environments (e.g., hypersaline sites and oil reservoirs) (Banat 1993; Abraham et al. 1998; Satpute et al. 2010a; Ibacache-Quiroga et al. 2013; Yan et al. 2014).

Detection methods for biosurfactant and bioemulsifier-producing microorganisms

High throughput techniques are required for rapid screening of potential biosurfactant-producing microorganisms in order to discover novel biosurfactant and/or biosurfactant-producing microorganisms (Maneerat 2005; Satpute et al. 2010a). Due to diverse functional and structural properties, the use of single screening method for the selection of biosurfactant-producing microorganisms has been very challenging in providing accurate and reliable results. Hence, the need for adoption of multiple screening tests concurrently for screening for maximum number of biosurfactant producers among the population of isolated microorganisms. The different screening methods commonly employed for biosurfactant/bioemulsifier production are based on surface or interfacial activity, or emulsification or foaming activities as described below:

Oil spreading assay

It is carried out by adding 10 µl of crude oil to the surface of 40 ml distilled water in a Petri dish to form a thin oil layer. Thereafter, 10 µl of culture supernatants are added on the oil coated water surface (Morikawa et al. 2000). Displacement of oil and formation of clear zone indicate the presence of biosurfactant in the culture supernatant sample. The diameter of clear zone on the oil surface correlates to surfactant activity. The oil spreading method is rapid, sensitive and reliable technique to detect biosurfactant production by diverse microorganisms (Youssef et al. 2004; Plaza et al. 2006; Ruggeri et al. 2009; Khopade et al. 2012; Manivasagan et al. 2014; Panjiar et al. 2015). In addition, it requires less sample volume and no specialized equipment, and can be employed when the activity and quantity of biosurfactant is low.

Surface and interfacial tension measurement

Surface tension is defined as a force per unit length exerted by a liquid when in close contact with another liquid or solid. In other words, it is a measure of the free energy per unit area that is linked with an interfacial or surface tension (Satpute et al. 2010a). Water molecules are bonded together by cohesive forces that form surface tension. The surface tension of distilled water is calculated to be 72 mN/m, addition of surfactants caused a reduction in the surface tension (Satpute et al. 2010a). Effectiveness of a biosurfactant depends on its ability to reduce the surface tension of water to less than 40 mN/m (Abdel-Mawgoud et al. 2010; Soberón-Chavez 2010). This is typical of a surfactin biosurfactant reported to reduce the surface tension of water to 27 mN/m (Cooper and Goldenberg 1987; Banat 1993). Similarly, Mulligan (2005) has reported a surfactin biosurfactant that decreased the surface tension of water to 25 mN/m, and the interfacial tension of water/hexadecane from 40 mN/m to 1 mN/m. In addition, a rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* has been reported to reduce the surface tension of water to approximately 30

mN/m and the interfacial tension of water/oil from 43 mN/m to almost 1 mN/m (Dusane et al. 2010). The direct measurement of surface tension of culture supernatants is carried out using tensiometer involving du Nuoy ring method, Wilhelmy plate method, maximum pull force method, or pendant drop methods. It is a precise and reliable technique for screening of biosurfactant production (Bodour et al. 2003; Youssef et al. 2004; Salihu et al. 2009; Patowary et al. 2017).

Emulsification activity

Among the functional properties of biosurfactant/bioemulsifier is emulsification, which involves dispersion of one liquid into another, resulting in the mixture of two immiscible liquids (Satpute et al. 2010a; Inès and Dhouha 2015). It is measured by calculating emulsification index (E_{24}), as described by Cooper and Goldenberg (1987). In this method, equal volume of hydrocarbon-based compounds and culture supernatants (2:2 v/v) is mixed together, vortexed for 2 min and allowed to stand for 24 h at room temperature. The E_{24} is calculated by dividing the height of the emulsion layer by the total height of liquid and expressed in percentage (%) (Cooper and Goldenberg 1987). Several authors have employed emulsification index as a technique for detection of biosurfactant/bioemulsifier production from microorganisms isolated from diverse environments (Das et al. 2008a; Jagtap et al. 2010; Chen et al. 2012; Zheng et al. 2012; Belgacem et al. 2015; Ndlovu et al. 2016).

Drop collapse method

Qualitative drop collapse assay for screening of biosurfactant/bioemulsifier-producing microorganisms was developed by Jain et al. (1991), and predominantly used for the measurement of surface tension of liquids. It is based on destabilization of liquid droplets by the surface-active compounds (Walter et al. 2010). The presence of biosurfactant causes the collapse or spread of the liquid droplets over a hydrophobic surface as a result of interfacial tension that exist between the liquid and the hydrophobic surface (Hsieh et al. 2004; Walter et al. 2010). A drop of broth culture or supernatant is added onto an oil-coated surface for 1 min. The spread or collapse of the drop sample suggests the presence of surfactants in the liquid sample (Jain et al. 1991; Bodour and Miller-Maier 1998; Walter et al. 2010). This is due to reduction in force or interfacial tension between the liquid and hydrophobic surface. However, if there are no biosurfactants in the culture sample, the drop remains stable as it is repelled by the hydrophobic surface. A low concentration of surfactants in the liquid droplets makes the drop collapse assay to be insensitive and inaccurate. This is due to the fact that, a high concentration of surfactant is needed for a noticeable collapse of the drop on oil surface (Satpute et al. 2008; Walter et al. 2010). The stability of the liquid droplets is influenced by the surfactant concentration, an indication that this method is reliable for indirect quantification of pure biosurfactant by measuring the drop size (Bodour and Miller-Maer 1998; Bodour et al. 2003). This

technique is easy to carry out, quick, and requires no sophisticated equipment and large sample volume. It has been employed for the screening of numerous microbes isolated from different environments for biosurfactant production (Bodour et al. 2003; Batista et al. 2006; Thavasi et al. 2011; Ibrahim et al. 2013; Panjiar et al. 2015; Belgacem et al. 2015; Ndlovu et al. 2016).

Cethyltrimethylammonium bromide agar plate method

The cethyltrimethylammonium bromide (CTAB) agar plate method is a semi-quantitative assay developed by Siegmund and Wagner (1991). It is used for the detection of extracellular glycolipids or other anionic surfactants. In this assay, the microorganisms are cultivated on a light blue mineral salt agar plate incorporated with cationic surfactant (CTAB) and the basic dye (methylene blue). The synthesis of anionic surfactant by the microorganisms is indicated by the formation of a dark blue, insoluble ion pair with CTAB and methylene blue. Biosurfactant producers are surrounded by dark blue halos. This technique is simple, specific for anionic biosurfactants and can be applied directly on agar plates or liquid broth using different substrates or temperatures (Pinzon and Ju 2009). However, CTAB is toxic and suppresses the growth of some microorganisms.

Blood haemolysis test

This is qualitative screening test for the detection of biosurfactant-producing microorganisms. It was developed by Mulligan et al. (1984) with the basic principle to determine the ability of biosurfactant to lyse red blood cells. Bacterial cultures are inoculated on blood agar plate, and then incubated at desired temperature for 2 d. The lysis of the blood cells and formation of colorless, transparent halo around the colonies is an indication of the presence of biosurfactant-producing organisms. This may be due to rupturing of cell membrane by the surface-active compounds (Satpute et al. 2010a). However, this method has been considered to be non-specific and unreliable (Youssef et al. 2004; Satpute et al. 2008; Fulazzaky et al. 2016).

Classification and chemical nature of biosurfactants

Microbial surface active compounds are commonly categorized based on their chemical components and microbial source (Pattanath et al. 2008; Banat et al. 2010; Vijayakumar and Saravanan 2015). According to Rosenberg and Ron (1999), microbial surfactants are grouped into two: low-molecular weight (glycolipids, lipopeptides, phospholipids) and high-molecular weight surfactants (polymeric and particulate surfactants) (Table 2).

Table 2 Major classes of biosurfactants

Class	Example
Glycolipid	Rhamnolipids Sophorolipids Trehalolipids
Lipopeptides and lipoproteins	Viscosin Serrawettin Polymyxin Gramicidin Subtilisin Surfactin Peptide-lipid
Fatty acids, neutral lipids and phospholipids	Fatty acids Neutral lipids Phospholipids
Polymeric surfactants	Emulsan Alasan Biodispersan Lipomanan Liposan Mannan lipid protein Carbohydrate-lipid-protein Protein PA
Particulate surfactant	Vesicles

Glycolipids

These are the most studied and well known biosurfactants, consisting of carbohydrate moiety connected to a long chain aliphatic acids or hydroxyaliphatic acid by a means of either ether or ester group. The carbohydrate domain occurs as mono-, di-, or tetrasaccharides including glucose, mannose, galactose, glucuronic acid, rhamnose or galactose sulphate. The fatty acid component possesses similar composition to that of phospholipid of the same microorganisms. The best known examples include rhamnolipids, trehalolipids and sophorolipids. However, the best-studied glycolipids are rhamnolipids, mannosylerythriol lipids, trehalose lipids, cellobiolipids, and sophorolipids. Rhamnolipids are a special class of best-studied glycolipids produced by *Pseudomonas* sp. (Chong and Li 2017; Ozdal et al. 2017), and consist of linkage of one or two molecules of rhamnose and one or two molecules of β-hydroxydecanoic acid. In this case, the OH group of one of the acids is connected to the reducing terminal of the rhamnose *via* a glycosidic bond whereas, the OH group of the second acid is involved in ester formation (Muthusamy et al. 2008).

Sophorolipids are a class of glycolipid biosurfactants secreted mainly by yeasts such as *Torulopsis bombicola*, *Torulopsis petrophilum* and *Torulopsis apicola* (Rahman and Gakpe 2008). They composed of a dimeric sophorose sugar linked to a long-chain hydroxyl fatty acid through a glycosidic bond. They occur in two types: acidic (non-lactone) and lactonic sophorolipids. The hydroxyl fatty acids moiety of the acidic sophorolipids consist of a free carboxyl acid functional group while that of lactonic sophorolipids has a macrocyclic lactone rings with the 4-hydroxyl group of the sophorose by intramolecular esterification. Both lactonic and acidic sophorolipids reduced the interfacial tension between *n*-hexadecane and water from 40 to 5 mN/m with a remarkable stability in pH and temperature (Desai and Banat 1997).

Mannosylerythritol lipids (MELs) are yeast glycolipids and one of the major biosurfactants produced by *Pseudozyma antarctica*. It consists of a mixture of four different components: MEL-A, MEL-B, MEL-C and MEL-D (Kitamoto et al. 1990; Morita et al. 2015). The backbone of these molecules is a mannose-erythritol disaccharide on which long-chain fatty acid chains are acetylated. Other MEL producers include *Pseudozyma parantarctica* (Morita et al. 2012), *Kurtzmanomyces* sp. I-11 (Kakugawa et al. 2002), and *Ustilago scitaminea* NBRC 32730 (Morita et al. 2011).

Lipopeptides and lipoproteins

These are a group of biosurfactants with remarkable surface active properties. A well-studied cyclic lipopeptide is surfactin produced by *Bacillus subtilis* ATCC 21332. Surfactin consists of seven amino acids linked to a carboxyl and hydroxyl groups of a 14-carbon acid (Chen et al. 2015). It is regarded as the most effective biosurfactant due to its ability to reduce the surface tension of water to 27 mN/m at low concentration (Kakimuna et al. 1969). Similarly, surfactant BL-86 produced by *Bacillus licheniformis* has also been reported to reduce the surface tension of water to 27 mN/m, and the interfacial tension between water and *n*-hexadecane to 0.36 mN/m (Desai and Banat 1997). A most important feature of surfactin is its potential to lyse mammalian red blood cells and form spheroplasts (Satpute et al. 2008).

Fatty acids, phospholipids and neutral lipids

Large amounts of fatty acids and phospholipids are produced by many bacteria and yeast when cultured in a medium supplemented with *n*-alkane. The hydrophilic and lipophilic balance is directly proportional to the length of the hydrocarbon chain in their structures (Satpute et al. 2010a). Phosphatidylethanolamine synthesized by *Acinetobacter* sp. form optically clear alkanes-water microemulsions, and that produced by *Rhodococcus erythropolis* reduces the interfacial tension between hexadecane and water to less than 1 mN/m and CMC of 30 mg/L (Desai and Banat 1997).

Polymeric biosurfactants

The most common polymeric biosurfactants include alasan, liposan, lipomanan, emulsan, and some other polysaccharide-protein complexes. Emulsan is an efficient bioemulsifier for hydrocarbons in water at concentrations as low as 0.001% to 0.01% (Lang 2002; Hatha et al. 2007). Liposan is an extracellular water-soluble emulsifier produced by *Candida lipolytica*, and consist of 83% carbohydrates and 17% proteins. Such carbohydrate-protein complex is also produced by *Yarrowia lipolytica*. Others include mannan lipid protein (*Candida lipolytica*), protein PA (*Pseudomonas aeruginosa*). However, emulsan and biodispersan produced by *Acinetobacter calcoaceticus* are the best-studied examples, consisting of a heteropolysaccharide moiety covalently linked to fatty acids (Rosenberg et al. 1988).

Particulate biosurfactants

The particulate biosurfactants partition extracellular membrane vesicles, forming microemulsion, which plays a major role in alkane uptake by microbial cells. Vesicles of *Acinetobacter* sp. has diameter of 20-50 nm, buoyant density of 1.158 cubic g/cm, and consist of protein, phospholipids and lipopolysaccharides (Chakrabarti 2012; Vijayakumar and Saravanan 2015).

Biosurfactant production and influencing factors

Biosurfactants are secreted extracellularly or intracellularly by microorganisms such as bacteria, yeasts, and fungi (Banat et al. 2014). The production of biosurfactant compounds occurs on water soluble and water insoluble substrates by *de novo* pathway and/or assembly from other substrates, and this may consequently vary in structure or production domain within the cell depending upon the substrate composition (Gautam and Tyagi 2006; Satpute et al. 2010b). Factors such as appropriate selection of microbial strains, substrate type, and fermentation technology play a vital role for a cost-effective and large-scale production of biosurfactants (Marchant and Banat 2012b; Marchant et al. 2014). Different growth media are utilized for effective biosurfactant production. In findings by Arima *et al*. (1968), low yields (~100 mg/L) of surfactin was reported from complex media such as nutrient broth and Luria Bertani broths. High yields of surfactin production by *Bacillus subtilis* strain when cultivated in mineral salt medium (MSM) has been reported (Yeh et al. 2005). These defined media have proved more effective for the production of biosurfactants (Wei et al. 2004). It consists of MSM supplemented with water soluble carbon sources such as glucose, sucrose, glycerol or water-insoluble carbon sources including blended gasoline, wheat bran, olive oil, hydrocarbons etc. (Prabhu and Phale 2003; Cunha et al. 2004). Govindammal and Parthasarathi (2013) investigated the influence of glucose, petroleum based substrates, waste fried vegetable oil, and coconut oil cake on biosurfactant production by *Pseudomonas fluorescence* MFS03

isolated from mangrove forest soil. Vegetable oil and coconut oil were recorded as reliable substrates for biosurfactant production. Similarly, Sim et al. (1997) studied the effect of vegetable oils (canola and soybean oils) and glucose on rhamnolipid biosurfactant production by *Pseudomonas aeruginosa* UW-1. Results recorded showed a 10-12-fold increase in the yield of rhamnolipid when vegetable oil rather than glucose was utilized as the sole carbon source. As a result, various authors (Desai and Banat 1997; Banat et al. 2000; Banat et al. 2014; Satpute et al. 2017) have suggested the use of vegetable oil as inexpensive and renewable substrates for biosurfactant production. Vegetable oil represents one of the first substrates reported for high yields of biosurfactants (Raza et al. 2007; George and Jayachandran 2013). Several authors (Abouseoud et al. 2008; Kiran et al. 2010a; Noudeh et al. 2010) have reported the use of olive oil as the best carbon source amongst the studied carbon sources for biosurfactant production.

Nitrogen sources form the second most essential supplement for the production of biosurfactants by microorganisms (Santos et al. 2016). Different organic (urea, yeast extract, peptone, tryptone, corn steep liquor) and inorganic (sodium nitrate, potassium nitrate, ammonium sulphate) nitrogen sources have been used in the production of biosurfactants (Abdel-Mawgoud et al. 2010). Santa Anna et al. (2002) investigated the importance of nitrogen source for the production of biosurfactant by *Pseudomonas aeruginosa* cultivated in MSM containing 3% glycerol. Sodium nitrate was found to be more effective than ammonium sulphate. High yields of sophorose lipids by *Torulopsis bombicola* and *Candida bombicola* have been obtained when using yeast extract and urea as the nitrogen sources (Deshpande and Daniels 1995). Ammonium nitrate and yeast extract have been reported as best nitrogen source for high yields of mannosylerythritol lipids by *Candida* sp. SY16, *Candida lipolytica* and *Candida glabrata* (Kim et al. 1999; Sarubbo et al. 2006, 2007; Rufino et al. 2007, 2008). Nitrogen limitation has been reported for improvement of biosurfactant production (Abu-Ruwaida et al. 1991). Patel and Desai (1997) observed that biosurfactant yield was enhanced under nitrogen limiting conditions. In addition, trace elements including Mg^{2+}, K^{+}, Mn^{2+} and Fe^{2+} act as key factors for the multi-enzyme systems associated with biosynthetic pathways of biosurfactant production (Sen and Swaminathan 2004; Wei et al. 2007; Chen et al. 2015**)**.

Kinetics of growth and biosurfactant production

The kinetics of biosurfactant production exhibit some considerable variations among different systems. Kinetic parameters are categorized as follows: (a) growth-associated production; (b) production under growth-limiting conditions; (c) production by resting or immobilized cells, and (d) production with precursor supplementation (Desai and Banat 1997). The interaction of various fermentation conditions in a complex way affects the kinetics of biosurfactant production. In growth-linked production, parallel relationships are noted between cell growth, substrate utilization and biosurfactant production. Zheng et al.

(2012) identified a novel bioemulsifier by *Aeribacillus pallidus* YM-1 cultivated in a MSM supplemented with 2.0% glucose as carbon source. Maximum cell growth (OD_{600} = 2.42 ± 0.02) corresponding to a significant increase in emulsifying activity (60 ± 1%) was achieved during exponential growth phase, an indication that production kinetics of the bioemulsifier was primarily growth-associated. Gudiña et al. (2015) studied bioemulsifier production by a novel *Paenibacillus* sp. #510 grown in MSM added with paraffin under aerobic conditions. Bioemulsifier production was found to be growth-linked, as parallel relationship was recorded between biomass production and emulsifying activity. Khopade et al. (2012) studied the kinetics of biosurfactant production from a marine *Streptomyces* sp. B3 under batch culture conditions. Biosurfactant production and surface tension were found to be growth depended. Similar profiles have been reported by several authors (Franzetti et al. 2008, 2012; Shavandi et al. 2011; Colin et al. 2013) have reported similar profiles.

Production under growth-limiting conditions is attributed to a drastic increase in the biosurfactant yield because of the limitation of one or more medium components. Several authors have reported overproduction of biosurfactants by *Pseudomonas* sp. when the cultures attain stationary growth phase due to deficient of nitrogen and iron (El-Sheshtawy et al. 2015; White et al. 2013; Lima et al. 2017).

In the case of production kinetics of biosurfactant by resting or immobilized cells, there is no cell growth; the cells continue to utilize the carbon source for biosurfactant synthesis (Desai and Banat 1997). This is noteworthy in the synthesis of rhamnolipid by *Pseudomonas* sp. (Onwosi and Odibo 2013; Lotfabad et al. 2017), glycolipid by *Ochrobactrum intermedium* (Ferhat et al. 2017), and lipopeptide surfactin by *Bacillus subtilis* ATCC 21332 (Chtioui et al. 2010).

Supplementation of growth medium with biosurfactant precursors induces both qualitative and quantitative changes in the metabolite. Increased biosurfactant production with yields of about 120-150 g/L following addition of lipophilic compounds to the fermentation medium has been reported (Lee and Kim 1993). Similarly, enhanced biosurfactant production from *Pseudomonas aeruginosa* MTCC 2297 and *Bacillus subtilis* ATCC 6633 through supplementation of the culture medium with sugars or sugar byproducts has also been recorded (Panesar et al. 2011; de Sousa et al. 2014).

Recovery and purification of biosurfactants

The different approaches employed for extraction and purification of biosurfactant compounds affect the production cost, and these vary according to the microbial growth production process and the physicochemical characteristics of the biosurfactant(s) produced (Shaligram and Singhal 2010). Downstream processing costs of biotechnological products account for approximately 70-80% of the total

production costs, making commercial production of biosurfactants quite costly (Santos et al. 2016). For instance, in certain applications such as cosmetics, medicine and food industries, biosurfactant production is only feasible on a small scale, since advance techniques required for separation of the biomolecules are not economical on a large scale. Hence, the use of crude biosurfactant preparations for environmental applications is preferable, since such biosurfactants do not require homogenous purity. The methods commonly used for effective recovery and/or purification of biosurfactants are influenced by the ionic charge, solubility and location of the synthesized surfactant compounds relative to the producing organisms (Helmy et al. 2011; Marchant and Banat 2012a). These methods include the use of organic solvents (butanol, ethyl acetate) or their combinations (chroloform-methanol, dichloromethane-methanol), and constitute the most widely used technique for biosurfactant downstream processing. However, this approach is liable to some limitations including increased production costs, use of large volume of organic solvents, and high toxicity of organic solvents. Other methods for recovery of biosurfactants involve precipitation with ammonium sulphate; centrifugation, and adsorption discussed below:

Acetone precipitation

In this process, cell-free culture supernatant is mixed with ice-cold acetone followed by incubation at 4 °C to recover the precipitate emulsifier. Several authors have employed this approach for the recovery of biosurfactants and bioemulsifiers (Sharma et al. 2015; Ferhat et al. 2017).

Ethanol precipitation

Similar to acetone, ethanol is regarded as a common solvent for extraction of crude bioemulsifier from the culture supernatant. The obtained cell free culture supernatant after centrifugation is precipitated using cold ethanol. This has been proved to be the most effective method for the precipitation of emulsifier from *Acinetobacter calcoaceticus* subsp. *anitratus* SM7 in comparison to other methods (Phetrong et al. 2008).

Ammonium sulphate precipitation

This method was developed by Rosenberg et al. (1979) for extraction of bioemulsifier from *Arthrobacter* RAG-1, and is employed for precipitation of high molecular weight bioemulsifiers including emulsan and protein rich biodispersan. Ammonium sulphate at initial concentration of 30% is added directly to the culture broth followed by incubation overnight. The obtained precipitate is further re-suspended in ammonium sulphate until a saturation level is reached. The resultant pellet was centrifuged and extracted with solvents. The obtained product is further purified and dialyzed. Depending on the type of bioemulsifier to be precipitated, different concentrations of ammonium sulphate have been used. Bach et al. (2003) added 60% ammonium sulphate to *Acinetobacter venetianus* RAG-1 cell free culture supernatant whereas, Toren

et al. (2001, 2002) precipitated alasan with 65% ammonium sulphate. In another study, Youssef et al. (2005) obtained a lipopeptide biosurfactant from *Bacillus* sp. by precipitation of obtained culture supernatant with 40% ammonium sulphate.

Acid precipitation

This method is based on the principle of hydrolysis of culture supernatant using concentrated HCl until a pH of 2.0 is attained. At such lower pH, protonated biosurfactants become insoluble and precipitate (Mukherjee et al. 2006; Gudiña et al. 2012). The precipitated biosurfactant is recovered by centrifugation after incubation overnight at 4 °C. The resultant pellet is dried under vacuum and further extracted using various solvents (chloroform, methanol ethyl acetate) (Haba et al. 2000; Thaniyavarn et al. 2003; Nitschke and Pastore 2006; Abbasi et al. 2012; Aparna et al. 2012). This method is simple and require less time for recovery of crude biosurfactant (Satpute et al. 2010a).

Dialysis and lyophilization

This is carried out by dissolving biosurfactant/bioemulsifier (precipitate) samples in sterile distilled water and dialyzed against double distilled water using a seamless cellulose tubing dialysis bag of a particular pore size. The obtained dialysate is then stored at 4 °C in an airtight container for further use. Bioemulsifier from *Acinetobacter calcoaceticus* BD4 and BD413 was recovered by ammonium sulphate precipitation. Further purification was carried by dialysis against cold distilled water followed by lyophilization of obtained product (Kaplan and Rosenberg 1982). This technique allows simple, rapid and cost-effective purification of biosurfactants.

Foam fractionation

This is a solvent-free technique used for recovery of biosurfactant compounds that are adsorbed to air bubbles in the fermentation medium. The production of biosurfactant involves continuous foam formation resulting from high surface active of the biomolecules. The foam-containing biosurfactant is collected from the fractionation column and acidified by precipitation using concentrated HCl to lower the pH to 1.0-2.0. The precipitated sample can further be extracted using organic solvent (Noah et al. 2002). This method possesses some advantages including prevention of product accumulation that could suppress biomass growth and product formation; facilitates prolonged biosurfactant production in fed-batch or continuous mode systems, and prevent biosurfactant denaturation due to their small size and simple structure (Rangarajan and Sem 2013).

Adsorption and desorption

The recovery of some biosurfactant molecules occurs by adsorption and desorption from Amberlite XAD 2 or 16 polystyrene resins. In this method, the biosurfactants are adsorbed on polymer resins followed by desorption with organic solvents for the collection of purified products (Abalos et al. 2001; Dubey et al. 2005). This technique is a rapid, one-step recovery process with a highly purified biosurfactant obtained. Adsorption-desorption of biosurfactant on wood-based activated carbon offers a continuous recovery of biosurfactant from fermentation broth and avoids end product inhibition (Dubey et al. 2005).

Ultrafiltration

This is a low pressure-driven process employed to concentrate and purify biosurfactants using amicon filter paper or hollow fiber cartridges. Biosurfactants are recovered from culture broth by ultrafiltration with 30 kDa molecular weight cut-offs (MWCO), which allows passage of small molecules (such as salts, amino acids, other metabolites) while retaining macromolecules of molecular diameter higher than MWCO of the membrane. The biosurfactant forms micelles at above CMC level, and these are collected in polymeric membranes. Several authors have reported on the use of ultrafiltration for the recovery of purified biosurfactants (Lin and Jiang 1997; Zinjarde et al. 1997; Bonilla et al. 2005). This technique is fast, simple, yields high purity biosurfactants and requires no chemicals.

Preparative thin layer chromatography

In preparative thin layer chromatography, the biosurfactants are purified by applying on a silica (stationary phase)-coated glass plate of varying thickness, and then allowed to run in a solvent system (mobile phase) (Satpute et al. 2010a; Biniarz et al. 2017). Bands of the sample are formed based on the affinity of the sample's components with the stationary and mobile phases. These bands are visualized under UV light (non-destructive approach) or using different solvents (destructive method). The obtained bands are further scraped and extracted with solvents. This method is versatile for detection of quality and purity of biosurfactants.

Ion exchange chromatography

Biosurfactants with net charge (e.g. negatively charged rhamnolipids) at higher pH are attached to ion-exchange resins and are eluted with buffer. The biosurfactant is released from the resin following the addition of a minimum of 0.6 M NaCl to the buffer. This process can be repeated several times using reusable resin in order to ensure a complete removal of salt from the biosurfactants (Matsufuji et al. 1997; Satpute et al. 2010a).

Potential applications of biosurfactants/bioemulsifiers

In recent years, much attention has been given toward biosurfactants due to their wide range of functional properties and diverse synthetic capabilities of microorganisms. Of utmost significant is the environmental acceptability of these surface-active compounds, as they are easily biodegradable with lower toxicity when compared to synthetic surfactants. These exceptional properties of biosurfactants allow their utilization and possible substitute of chemically synthesized surfactants in a wide range of biotechnological applications including petroleum, agriculture, food processing, cosmetics, detergents, leather, textile, paper and pharmaceutical industries (Rodrigues et al. 2006; Banat et al. 2010; Gudiña et al. 2016). Furthermore, biosurfactants are used for the recovery of oil residue from storage tank, clean-up of oil spills and bioremediation of contaminated soil and water (Sobrinho et al. 2013; Silva et al. 2014a). A summary of the potential applications of biosurfactants in various industries is presented in Table 3, while the major biotechnological applications are discussed in details below:

Bioremediation

The discharge of contaminants such as hydrocarbons into the environment represents one of the crucial causes of global pollution and has become a major issue of concern in both industrialized and developing countries (Lobrero et al. 2012; Luna et al. 2013). This is typical of the largest oil spills in the world, which occurred in the Gulf of Mexico in 2010, following the explosion of oil off the coastline of the states of Louisiana and Mississippi (USA), resulting in the release of an estimated 3-4 million barrels of oils spilled (Rocha et al. 2013).

Different physicochemical approaches including gravity separation, chemical coagulation, cyclone separation have been developed for the treatment of oil-contaminated sites (Abdelwahab et al. 2009; Diya'udeen et al. 2011; Padaki et al. 2015). However, these techniques are often expensive and inefficient. Therefore, biological methods are considered more suitable due to their less hazardous and more selectivity to specific reactions (Assadi and Tabatabaee 2010). It involves the use of natural degradation capacity of microorganisms to eliminate or neutralize pollutants from a contaminated site by conversion of the contaminants into less toxic compounds or complete conversion of such substances into carbon dioxide and water (Santos et al. 2016). The use of biosurfactant emerges as an alternative approach for enhancing the dispersal and solubility of contaminants in the aqueous phase, and increase the bioavailability of the hydrophobic substrates to microorganisms, with subsequent removal of such pollutants through biodegradation, thus eliminating the need for additional process and results in lesser operational costs (Kuyukina et al. 2005; Assadi and Tabatabaee 2010; Aparna et al. 2011; Daverey and Pakshirajan 2011; Damasceno et al. 2012; Olkowska et al. 2012).

Table 3 Some potential biotechnological applications of biosurfactants

Industry	Application	Role	Reference
Detergent	Washing detergents	Additive for improved performance and stain removal	Vijayakumar and Saravanan (2015)
Medicine	Pharmaceutical and therapeutics	Anti-bacterial, anti-adhesive, anti-fungal, anti-viral, anti-mycoplasma, anti-tumoral, anti-inflammatory, anti-coagulant, anti-cancer agents	Banat et al. 2010, 2014; Mnif and Ghribi (2015); Gudiña et al. 2016
Agriculture	Biocontrol; Biofertilizers	Emulsifying agents, dispersing agents, spreading agents and wetting agents; Improvement of soil quality, plant pathogen elimination, facilitation of biocontrol of microbes, enhanced bioavailability of nutrients for beneficial plant-associated microorganisms	Sachedev and Cameotra (2013)
Cosmetics	Health and beauty products	Emulsifying agents, foaming agents, spreading agents, wetting agents, antimicrobial agents, cleansers	Vijayakumar and Saravanan (2015)
Nanotechnology	Nanoparticle synthesis	Stabilization, adsorption, dispersion and emulsification	Biswas and Raichur (2008); Reddy et al. 2009; Farias et al. 2014
Environment	Bioremediation of contaminated soil and water, clean-up of oil spills, microbial-enhanced oil recovery	Emulsification, dispersion, desorption, solubilization; Anti-corrosive agents, wetting agents, foaming agents, soil flushing, surface tension reduction	Banat et al. 2010; Pacwa-Plociniczak et al. 2011; Silva et al. 2014a
Food	Emulsification, de-emulsification, functional ingredient	Stabilization of emulsion, phase dispersion, lowering of surface and interfacial tension; Improvement of texture, shelf life and consistency; Emulsifiers, control of fat globule agglomeration	Campos et al. 2013

Numerous studies have demonstrated the potential applications of biosurfactant/bioemulsifier in environmental decontamination (Pornsunthorntawee et al. 2008; Millioli et al. 2009; Hu et al. 2013; Chaprão et al. 2015). Maier and Soberon-Chavez (2000) reported that the addition of rhamnolipid to environments enhances the degradation of hydrocarbon-based pollutants. The use of rhamnolipid and surfactin to enhance the degradation of diesel-contaminated soil and water has been reported (Whang et al. 2008). Addition of the two biosurfactant compounds and their producer organisms (*Pseudomonas aeruginosa* J4 and *Bacillus subtilis* ATCC 21332) stimulates microbial growth, as a result of increased efficiency and rate of diesel biodegradation. Gusmão et al. (2010) assessed the potential of crude biosurfactants produced by *Candida glabrata* UCP 1002 in the remediation of soil-water hydrophobic

pollutants. Removal efficiency of 92.6% was recorded. In another study, a novel biosurfactant, denoted Lunasan produced by *Candida sphaerica* UCP 0995 was found to remove 95% of motor oil from contaminated site (Luna et al. 2011). *Gordonia* sp. BS29 cultivated in hydrophobic substrate as sole carbon source has been found to synthesize bioemulsan which effectively degrade crude oil, polycyclic aromatic hydrocarbons and other recalcitrant branched hydrocarbons from contaminated sites (Franzetti et al. 2008).

The addition of biosurfactants/bioemulsifiers serves as a promising strategy for the biotreatment of oily wastewater (de Almeida et al. 2016; Rahbari-Sisakht et al. 2017). Zhang et al. (2009) applied rhamnolipids for the treatment of oily wastewater in an aerated active sludge system. At 20 °C, rhamnolipids (11.2 mg/L) was found to increase the removal efficiency of oil from 17.7% (without rhamnolipids) to 63%. At 25 °C, the removal efficiency of oil was above 80% in the presence of rhamnolipids as compared to 22.3% without rhamnolipids. Similarly, rhamnolipid treatment (22.5 mg/L) for 24 h at 20 °C significantly increased the removal rate of oil from 24% (in the absence of rhamnolipids) to 92%. The authors therefore observed that enhanced removal of oils was due to improved solubility, and reduced interfacial tension of the rhamnolipids. Lima et al. (2011) assessed the removal of oily sludge from oil-contaminated sites using biosurfactants produced from five bacterial isolates. The biosurfactants led to a reduction in viscosity and enhanced the formation of oil-water emulsions leading to easier sludge pumping and emulsion breaking for better oil recovery. In another study, Yan et al. (2012) used a rhamnolipid produced by *Pseudomonas aeruginosa* F-2 for the reclamation of oil from oily sludge. Removal efficiency (91.5%) of oil was recorded from the field pilot-scale studies. Daverey and Pakshirajan (2011) evaluated the pretreatment of synthetic dairy wastewater using sophorolipid biosurfactant produced by *Candida bombicola*. The results showed COD removal efficiency (93%) after 96 h of operation. Furthermore, Cosmann et al. (2017) employed a surfactin biosurfactant produced by *Bacillus subtilis* LB5a for the anaerobic treatment of poultry slaughterhouse effluent. Results obtained revealed no inhibition of microbial consortium of the anaerobic sludge with consequent soluble COD (SCOD) and O & G removal efficiencies of 80% and 70%, respectively along with a promising specific methane yield. Daverey and Pakshirajan (2011) evaluated the pretreatment of synthetic dairy wastewater using sophorolipid biosurfactant produced by *Candida bombicola*. The results showed COD removal efficiency (93%) after 96 h of operation. In addition, Cosmann et al. (2017) employed a surfactin biosurfactant produced by *Bacillus subtilis* LB5a for the anaerobic treatment of poultry slaughterhouse effluent. Results obtained revealed no inhibition of microbial consortium of the anaerobic sludge with consequent soluble COD (SCOD) and O & G removal efficiencies of 80% and 70%, respectively along with a promising specific methane yield.

Increase in the levels of heavy metals and radionuclides in soil in many industrialized countries poses serious threats to the ecosystems and human health through either food chain or direct exposure to

contaminated soil and water (Chakraborty and Das 2014; Mao et al. 2015). The heavy metals adsorb to the soil surface in the form of ions or the precipitation of metal compounds. They are removed from soil through surfactant-associated complexation and ion exchange (Ochoa-Loza et al. 2001; Swarnkar et al. 2012). Dahrazma and Mulligan (2007) assessed the efficacy of rhamnolipid biosurfactant for the removal of heavy metals (Cu, Ni and Zn) from sediments obtained from the Lachine Canal in Canada in a continuous flow configuration system. Rhamnolipid concentration (0.5%, v/v) led to removal efficiencies of Cu (37%), Ni (27%) and Zn (13%). Addition of 1% NaOH further increased (4-fold) the removal of heavy metals from the system. Biosurfactant compounds synthesized by *Pseudomonas* sp. and *Alcaligenes* sp. have been employed for the floatation and removal of calcite and scheelite. Recovery rates of 95% ($CaWO_4$) and 30% ($CaCO_3$) were recorded in comparison to conventional surfactant, found to be incapable of separating these two minerals (Nitschke and Pastore 2002). Similarly, Juwarkar et al. (2007) investigated the potential of rhamnolipid for the removal of Cd^{2+} and Pb from artificially contaminated soil samples. The rhamnolipid compound was found to remove both free and weakly bound Cd^{2+} and Pb from the tested soil.

Food industry

In food industries, biosurfactants have several promising applications including formation and stabilization of emulsions. Emulsification is vital for the formation of consistency and texture in foods; phase dispersion and solubilization of aromas (Campos et al. 2013; 2014). Generally, emulsifiers in food products stabilize emulsion by controlling agglomeration of fat globules, stabilization of aerated systems, improvement of texture and shelf life of starch-containing products, alteration of rheological properties of wheat dough and enhancement of consistency and texture of fat-based products (Nitschke and Pastore 2002; Campos et al. 2013). Enhancement in the stability of dough, volume, texture and conservation of bakery products following addition of rhamnolipid biosurfactants has been reported (Van Haesendonck and Vanzeveren 2004). In addition, bioemulsifier produced by *Candida utilis* is used in processed salad dressing (Campos et al. 2015). Mannoprotein secreted by *Saccharomyces cerevisiae* has been reported to stabilize water/oil emulsions in cookies, mayonnaise and ice cream (Shepherd et al. 1995; Torabizadeh et al. 1996).

Medicine

Several microorganisms have developed multi-drug resistance to commercially available antimicrobial agents, and this is associated with misuse or abuse of antibiotics. Thus, persistent and chronic infectious diseases emerged, constituting a serious public health concern (Coates et al. 2011). Furthermore, in the last decades, the discovery of new antibiotics has reduced significantly, due to challenges in identification of novel and hyperactive compounds with subsequent high costs needed for their development. Therefore,

there is an urgent need for a renewed interest in the development of novel antimicrobial drugs with wide spectrum activity.

Biosurfactants have been employed in various therapeutic applications due to their bactericidal, fungicidal, insecticidal and anti-viral activities as well as use as anti-fouling agents. Several biosurfactants displayed similar or better antimicrobial activity in comparison with conventional antibiotics. For instance, glycolipid biosurfactant produced by *Streptomyces* sp. MAB36 was found to exhibit similar inhibitory activity against *Aspergillus niger* and *Candida albicans* when compared with the conventional antifungal nystatin (Manivasagan et al. 2014). In another study, biosurfactant produced by *Nocardiopsis dassonvillei* MAD08 was reported to be more effective against *Escherichia coli* and *Staphylococcus epidermidis* than chloramphenicol (Selvin et al. 2009). In addition, sophorolipids from *Candida bombicola* was found to inhibit the growth of Gram-negative and Gram-positive bacteria with a minimum inhibitory concentration of approximately 30 and 1 mg/ml in a contact time of 2 and 4 h, respectively for *Escherichia coli* ATCC8739 and *Pseudomonas aeruginosa* ATCC 9027 as well as 6 and 1 mg/ml in a contact time of 4 h for *Staphylococcus aureus* ATCC 6358 and *Bacillus subtilis* ATCC 6633, respectively (Joshi-Navare and Prabhune 2013).

Furthermore, some biosurfactant compounds possessed a substantial anti-adhesive and anti-biofilm activity. Glycolipid biosurfactant synthesized by *Brevibacterium casei* MSA19 was found to remove pre-formed biofilms of all the tested pathogenic microorganisms at a concentration of 30 µg/ml (Kiran et al. 2010b). Similarly, Lunasan-producing *Candida sphaerica* was reported to inhibit adherence of *Pseudomons aeruginosa*, *Streptococcus agalactiae* and *Streptococcus sanguis* between 80% and 92% when the concentration was 10 mg/ml (Luna et al. 2011) whereas, biosurfactant Rufisan showed anti-adherent activity against most of the tested microorganisms including *Streptococcus agalactiae*, *Streptococcus mutans*, *Streptococcus mutans* NS, *Streptococcus mutans* HG, *Streptococcus sangui* 12, *Streptococcus oralis* J22 at a concentration above the CMC (0.3%) (Rufino et al. 2011).

Biosurfactants have been highlighted as potential anti-cancer agents by interfering with cancer progression processes. These biomolecules are involved in several intracellular molecular recognition steps consisting of signal transduction, cell differentiation and cell immune response etc. (Rodrigues et al. 2006). For instance, surfactin biosurfactants are commonly used as potential anti-cancer agent against many cancer cell lines (Sivapathasekaran et al. 2010; Gudiña et al. 2013; Dey et al. 2015). Iturin A produced by *Bacillus megaterium* strain has been found to significantly impair proliferation and inhibit the Akt signaling network resulting in apoptosis induction in breast cancer cells (MDA-MB-231 and MCF-7) (Dey et al. 2015). In addition, lipopeptide somocystinamide A secreted from cyanobacteria, *Lyngbya majuscula* demonstrated

noteworthy cytotoxicity against leukaemia, lung, breast and prostate cancer cells with IC_{50} values between 1.3 μM to 970 nM, based on the cancer model (Wrasidlo et al. 2008).

Cosmetic industry

Biosurfactants possess broad potential application in the cosmetic industry, owing to their multifunctional properties including emulsification, de-emulsification, foaming, water-binding capacity, and spreading and wetting, which allow them to be efficiently utilized in cosmetic products such as emulsifiers, solubilizers, antimicrobial agents, bath products like anti-dandruff shampoo, baby products, toothpaste etc. (Youssef et al. 2007; Shekhar et al. 2015). Biosurfactants are better skin compatible compared to their synthetic counterparts (Williams 2009). Lipopeptide biosurfactants with moisturizing and anti-wrinkle properties demonstrated low cytotoxic effects in human cells. Thus, they are employed as additives in dermatological products (Mandal et al. 2013). In addition, monoglyceride produced from *Pseudomonas fluorescens* is a commonly used surfactant in the cosmetic industry (McNeill and Yamane 1991).

Nanotechnology

The application of biosurfactant in nanotechnology and nanoparticle synthesis is a new development in the area of green chemistry (Kiran et al. 2010a; Martinez et al. 2014; Santos et al. 2016). Reddy et al. (2009) observed that silver nanoparticle synthesis could be stabilized for up to two months using surfactin. Similarly, biosurfactant produced by *Pseudomonas aeruginosa* has been used to stabilize silver nanoparticles in the liquid phase (Farias et al. 2014). In another study, glycolipid biosurfactant produced from *Brevibacterium casei* MSA 19 has been reported to stabilize silver nanoparticles (Kiran et al. 2010a).

Concluding remarks

The global market for microbial surface-active compounds has increased significantly over decades. This is due to the outstanding functional properties of these biomolecules, forming a foundation for exploration in bioremediation, cosmetics, food, medicine and nanotechnology. However, mass production of microbial surfactants formed a major drawback from economic perspective. Optimization of growth and fermentation parameters using cheaper renewable substrates coupled with robust downstream processing methods could pave the way for profitable and cost-effective surfactant production. In addition, the use of recombinant and mutant hyper-producing microorganisms could provide high yield surfactant production that would be beneficial for industrial and biotechnological applications.

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CHAPTER THREE

PRODUCTION, PROPERTIES AND POTENTIAL BIOTECHNOLOGICAL APPLICATIONS OF MICROBIAL PROTEASES -- A REVIEW

This chapter has been formatted for submission to the '3 Biotech'

Production, properties and potential biotechnological applications of microbial proteases- A Review

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Abstract Proteases are among the most important classes of hydrolytic enzymes, and occupy a pivotal position with respect to their applications in both physiological and commercial fields. They catalyze the cleavage of peptide bonds that exist between amino acid residues in proteins. Proteases are essential constituents of all forms of life, including plants, animals and microorganisms. However, microorganisms represent an attractive source due to their high productivity in a relatively short time, rapid growth, broad biochemical diversity, and susceptibility to genetic manipulation among others. Microbial proteases possess all the characteristics desired for their biotechnological applications, and constitute approximately 40% of the total worldwide production of enzymes. Extracellular protease production from microorganisms is strongly influenced by media components and other physical parameters. Exploitation of statistical experimental designs for optimization of process parameters for large-scale protease production is among the most recently developed methods for improving enzyme production from different microorganisms. The catalytic properties of microbial alkaline proteases help in the discovery of enzymes with high activity and stability over extreme temperature and pH for various biotechnological applications. The focus of the present review is to provide an updated overview of the fermentative production of microbial proteases as well as the various statistical approaches employed for the optimization of these biocatalysts. In addition, downstream purification and immobilization techniques coupled with biochemical properties of proteases for potential biotechnological applications were extensively discussed.

Keywords Proteases, Microorganisms, Microbial proteases, Alkaline proteases, Production, Applications

Introduction

Enzymes are biological macromolecules consisting of monomers of amino acids linked together by amide bonds. They are produced by living organisms, and act as catalyst to accelerate a specific biochemical reaction required to sustain life (Gurung et al. 2013). Enzymes are highly selective biocatalysts, which speed up both the rate and specificity of biological reactions by lowering the activation energy without

undergoing any permanent change (Aldridge 2013). The catalytic site of these macromolecules is contained within hydrophobic pockets, which determines their specificity for substrate (Singh et al. 2016a).

The global demands for industrial enzymes is estimated at 4.2 billion dollars in 2014 and anticipated to raise at a compound annual growth rate of about 7% from 2015 to 2020 to reach about 6.2 billion dollars (Enzyme Industrial Market 2015). Enzymes play a vital role in numerous biotechnological applications. Currently, the most commonly used (more than 75%) enzymes for commercial applications are hydrolases, which catalyze the hydrolysis of various natural molecules (Prakash et al. 2013). However, proteases are recognized as the leading enzyme, due to their versatility in industrial biotechnology (Gurung et al. 2013).

Proteases are the largest and complex group of enzymes that catalyze the breakdown of proteins by cleavage of peptide bonds that exist between amino acid residues in a polypeptide chain (Shankar et al. 2011; Ibrahim et al. 2015b). They constitute one of the most important group of industrial enzymes, accounting for more than 65% of the total industrial enzyme market (Sundararajan et al. 2011; Annamalai et al. 2014). Proteases occupy a key position with respect to their applications in both physiological and biotechnological fields including detergent, pharmaceutical, food and leather industries as well as in environmental pollution abatement (Sawant and Nagendran 2014; de Souza et al. 2015).

This review therefore focusses on the fermentative production of microbial proteases and provides insight on various statistical approaches used for optimization of process parameters for enhanced production of this biocatalyst. Techniques for downstream purification of proteases and their immobilization in/on appropriate support materials were further discussed. Catalytic properties of alkaline proteases for exploitation for various biotechnological applications were also discussed.

Sources of proteases

Proteases play a key physiological role in all living organisms. Thus, they are ubiquitous in nature and found in a wide variety of sources including plants, animals, and microorganisms (Rao et al. 1998).

Plant proteases

Some of the well-known proteases of plant origin include papain, bromelain, and ficin, extracted from *Carica papaya*, *Ananas comosus* and *Ficus carica,* respectively. They are commonly employed in food and pharmaceutical industries involving brewing, meat tenderization, milk coagulation, digestion, cancer and viral treatment as well as in the preparation of soluble protein hydrolysates. Keratinase is used for the production of lysine by digestion of hair and wool and for prevention of blocking of wastewater systems. However, despite their enormous applications, protease production from plant sources is influenced by land

availability for cultivation and climatic conditions for growth (Rao et al. 1998; González-Rábade et al. 2011).

Animal proteases

The most widely used animal-based proteases include trypsin, chemotrypsin, pepsin and rennin (Rao et al. 1998). Trypsin constitutes a major digestive enzyme found in intestine and responsible for the hydrolysis of food proteins, and as well serves as target for biocontrol of insect pests. In addition, it is used in the formulation of microbial growth media and in some specialized medical applications. Chymotrypsin is found in the pancreatic extract of animals. When in pure form, it is used for various diagnostic and analytical purposes, especially in the deallergenization of milk protein hydrolysates. Pepsin is an acidic protease found in the stomach of most vertebrates. Rennet is a pepsin-like protease produced as an inactive precursor in the stomach of all nursing mammals; its conversion to active renin is by the action of pepsin or autocatalysis. It is commonly used in the milk industry for the production of stable curd with good flavor (Rao et al. 1998).

Microbial proteases

Microbial proteases are among the most important and extensively studied hydrolytic enzymes since the beginning of enzymology (Gupta et al. 2002). They constitute 40% of the total worldwide production of enzymes (Haddar et al. 2009; Raval et al. 2014). The failure of plant and animal proteases to meet global demands has led to an increased interest in microbial proteases. They are produced by a large number of microbes including bacteria, fungi, yeasts and actinomycetes (Kumar and Takagi 1999). The various protease-producing microorganisms are presented in Table 1. The microorganisms represent an excellent source of proteases due to their rapid growth, broad biochemical diversity, ease of genetic manipulation and limited space requirement for cultivation. In addition, the microorganisms can be cultivated in large amounts in a relatively short time by an established fermentation process for the mass production of desired metabolite. Microbial proteases are commonly extracellular in nature, secreted directly into fermentation medium by the producing-organisms, thus shortening downstream processing of the enzyme when compared to plant and animal proteases (Gupta et al. 2002). They have a longer shelf life and can be preserved for a long period without significant loss of activity. However, of all the microbial sources, bacterial proteases are of particular interest due to their high catalytic activity and stability at optimal pH (8-12) and temperature (50-70 °C) and broad substrate specificity (Vijayalakshmi et al. 2011). These characteristics of bacterial proteases warrant their suitability for use in various biotechnological applications including detergent, leather processing, textile, food and feed industries as well as in waste management (Kumar and Takagi 1999; Singhal et al. 2012).

Table 1 Some protease-producing microorganisms

Microorganism	Reference
Bacteria	
Bacillus sp. CL18	Rieger et al. 2017
Geobacillus toebii LBT 77	Thebti et al. 2016
Pseudomonas fluorescens BJ-10	Zhang et al. 2015
Streptomyces sp. DPUA 1576	Silva et al. 2015
Vibrio mimicus VM 573	Mizuno et al. 2014
Lactobacillus helveticus M92	Beganović et al. 2013
Microbacterium sp. HSL10	Lü et al. 2014
Serratia marcescens RSPB 11	Bhargavi and Prakasham (2016)
Listeria monocytogenes	Shumi et al. 2004
Brevibacterium linens ATCC 9174	Rattray et al. 1995
Alteromonas sp.	Yeo et al. 1995
Halobacillus blutaparonensis M9	Santos et al. 2013
Staphylococcus epidermidis	Vandecandelaere et al. 2014
Yersinia ruckeri	Secades and Guijarro (1999)
Fungi	
Alternaria solani	Chandrasekan and Sathiyabama (2014)
Aspergillus niger DEF 1	Lanka et al. 2017
Penicillium sp. LCJ228	Benluvankar et al. 2015
Fusarium solani	Al-Askar et al. 2014
Rhizopus stolonifer	Liu and Huang (2015)
Trichoderma viridiae VPG12	Shivasharanappa et al. 2014
Mucor sp.	Alves et al. 2005
Beauveria sp.	Shankar et al. 2011
Cephalosporium sp. KSM 388	Tsuchiya et al. 1987
Yeasts	
Wickerhamomyces anomalus 227	Schlander et al. 2017
Metschnikovia pulcherrima 446	Schlander et al. 2017
Candida spp.	de Souza Ramos et al. 2015
Yarrowia lipolytica	Matoba et al. 1997
Rhototorula mucilaginosa KKU-M_{12C}	Hesham et al. 2017
Cryptococcus albidus KKU-M_{13C}	Hesham et al. 2017

Classification of microbial proteases

Microbial proteases are generally classified based on three major criteria namely; type of reaction catalyzed, evolutionary relationship with preference to structure and chemical nature of catalytic site (Barett 1994). They are also classified into three groups: acidic proteases, neutral proteases and alkaline proteases depending on optimal pH of activity. Based on site of action, they are divided into two major groups *viz*. exopeptidases and endopeptidases. Exopeptidases catalyze the cleavage of peptide bonds close to the amino or carboxyl moiety of the protein whereas, endopeptidases cleave peptide bonds away from the termini of the substrate (Rao et al. 1998; de Souza et al. 2015). The exopeptidases are further classified as amino- and

carboxypeptidases, depending on their site of action at the amino or carboxyl terminus of protein. The endopeptidases are sub-divided into four groups: serine proteases, aspartic proteases, cysteine proteases, and metalloproteases, based on their catalytic mechanisms (Li et al. 2013).

Serine proteases

Serine proteases are characterized by the presence of a serine residue in the active site, and are generally recognized by irreversible inhibition by diisopropyl fluorophosphates (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone. In addition, thiol reagents such as *p*-chloromercuribenzoate (*p*CMB) also inhibit some of the serine proteases. These proteases are active at neutral and alkaline pH, with optimum pH ranging between 7-11. They have broad substrate specificities and low molecular weight ranging between 18 to 35 kDa (Rao et al. 1998; Ellaiah et al. 2002).

Cysteine proteases

Cysteine proteases, also known as thiol proteases consist of about 20 families, the activity of which is influenced by a catalytic dyad comprising of cysteine and histidine. They are sensitive to sulphydryl reagents, including *p*CMB, iodoacetic acid, iodoacetamide, heavy metals, but are not affected by DFP and metal-chelating agents. In addition, they are active in the presence of reducing agents such as HCN or cysteine, dithiothreitol, and ethylene diaminetetraacetic acid (EDTA). Cysteine proteases have neutral pH optimal or maximally active at acidic pH (Rao et al. 1998).

Metalloproteases

Metalloproteases are the most diverse catalytic form of protease, which depend on divalent metal ions for their activity (Barett 1995; Vranova et al. 2013). They have optimal pH in the range of 5.0 to 9.0, and are sensitive to chelating agents, such as EDTA, but are unaffected by serine protease inhibitors or sulphyldryl agents. Numerous EDTA-inhibited enzymes are activated by ions, such as zinc, calcium, and cobalt. Most of the bacterial and fungal-derived metalloproteases are zinc-containing enzymes, consisting of one atom of zinc per molecule of enzyme. The zinc atom is important for enzyme activity. The stability of the protein structure is enhanced by calcium ions (Ellaiah et al. 2002).

Aspartic proteases

Aspartic proteases, also known as acidic proteases are endopeptidases characterized by the presence of aspartic acid residues for catalytic activity (Rao et al. 1998). They are commonly found in fungi, but seldom found in bacteria or protozoa. They are generally divided into two sub-classes viz. pepsin-like enzyme synthesized by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora*, and rennin-like enzymes produced by

Endothia and *Mucor* sp. Most aspartic proteases show maximal activity at low pH (pH 3-4) with isoelectric points and molecular masses in the range of pH 3.0-4.5 and 30-45 kDa, respectively (Rao et al. 1998; Srilakshmi et al. 2015). The active-site (aspartic acid residue) is situated within the motif Asp-Xaa-Gly, where Xaa represents Ser or Thr. Inhibitors such as pepstatin and diazoketone molecules including diazoacetyl-DL-norleucine methyl ester and 1,2-epoxy-3-(*p*-nitrophenoxy) propane inhibit aspartic protease activity in the presence of copper ions (Singh et al. 2016b). They are specific against aromatic or bulky amino acid residues on both sides of the peptide bond.

Proteases production and influencing factors

Microorganisms generally produce protease extracellularly by submerged fermentation and to a lesser extent by solid-state fermentation processes during post-exponential or stationary growth phase (Mukherjee et al. 2008; Reddy et al. 2008). Among the bacterial genera that have been reported for extracellular alkaline protease production include, *Alteromonas*, *Brevibacterium*, *Microbacterium*, *Pseudomonas*, *Streptomyces*, *Vibrio*, *Bacillus*, *Xanthomonas* (Jisha et al. 2013). However, members of the genus *Bacillus* have been reported as main source for large scale production of alkaline proteases (Navaneeth et al. 2009; Queiroga et al. 2012). Protease production from microorganisms is constitutive or partially inducible in nature, and the type of substrate utilized in the fermentation medium mostly influences their synthesis. Selection of appropriate inducible substrate and microorganisms is paramount for the production of desired product (Beg et al. 2002; Anandan et al. 2007; Bhunia et al. 2012). Under most culture conditions, extracellular protease production by microorganisms is strongly influenced by media components including variation in C/N ratio, presence of some readily metabolizable sugars, such as glucose, and metal ions (Beg et al. 2002; Bhunia et al. 2012). In addition, protease synthesis is also greatly affected by rapidly metabolizable nitrogen sources, such as amino acids in the fermentation medium (Gupta et al. 2002). The alkaline protease consists of 15.6% nitrogen; its production is dependent on the presence of both carbon and nitrogen sources in the culture medium (Kole et al. 1988). Various authors have reported increased yields of protease production by addition of different carbon and nitrogen sources (Sharma et al. 2017). Sharma et al. (2015) recorded maximum protease production by a bacterial strain AKS-4 when glucose was used as a carbon source in the growth media at a concentration of 1% (w/v), resulting in a maximum activity of 59.10 U/ml. In another study, Sharma et al. (2014) used various carbon sources including glucose, lactose, galactose and starch for protease production by *Bacillus aryabhattai* K3. Maximum protease production (622.64 U/ml) was observed in the presence of lactose (10 g/L).

Although complex nitrogen sources are commonly utilized for alkaline protease production, the requirement for a particular nitrogen supplement differs from one organism to another (Kumar and Takagi 1999; de Souza et al. 2015). In most microorganisms, both organic and inorganic nitrogen sources are metabolized to produce amino acids, nucleic acids, proteins and other cell wall components (Kumar and Takagi 1999; Ellaiah et al. 2002). Several authors have employed organic (simple or complex) and inorganic nitrogen sources for enhancement of protease production. These nitrogen sources have regulatory effects on protease synthesis. Kumar et al. (2014) studied the effect of organic and inorganic nitrogen sources on protease production by *Marinobacter* sp. GA CAS9. Results obtained revealed that organic nitrogen sources induced higher protease production than inorganic nitrogen sources, with maximum protease production (249.18 U/ml) recorded in the presence of beef extract. Badhe et al. (2016) studied the influence of nitrogen sources including ammonium nitrate, ammonium chloride, ammonium sulphate, yeast extract, potassium nitrate, and sodium nitrate on extracellular protease production by *Bacillus subtilis*. Yeast extract was found as the best nitrogen source to stimulate maximum protease production. Urea and sodium nitrate have been reported as the best organic and inorganic nitrogen sources, respectively for extracellular protease production by *Bacillus licheniformis* ATCC 12759 (Akcan 2012).

Increase in the concentration of carbon and nitrogen sources in the fermentation medium suppresses protease production (Kumar and Takagi 1999; Ellaiah et al. 2002). Inhibition of protease synthesis by *Pseudomonas aeruginosa* MCM B-327 in soybean-tryptone media following addition of glucose (95%) and fructose (60%) has been observed (Zambare et al. 2011). Mehta et al. (2006) recorded a repression in alkaline protease production due to increase in the concentration of glucose, peptone, yeast extract and amino acids in the fermentation media.

Several bioprocess parameters including pH, temperature, aeration, inoculum density, agitation speed and incubation period also influence protease production (Puri et al. 2002; Srividya and Mala 2011; Pathak and Deshmukh 2012). These parameters are essential to promote the growth of microorganisms for protease production. Slightly acidic medium pH (6.3-6.5) has been found as optimum for protease production by *Bacillus* sp. MIG (Gouda 2006) and *Bacillus cereus* SIU1 (Singh et al. 2010). Maximum protease production by *Bacillus subtilis* NS and *Pseudomonas fluorescens* was recorded when the initial pH of the fermentation media was 9.0 (Nisha and Divakaran 2014; Vinoth et al. 2014). Higher initial pH 12.0 for *Bacillus cereus* S8 (Lakshmi et al. 2014), 10.5 for *Bacillus circulans* (Jaswal et al. 2008) and 10.7 for *Bacillus* sp. 2-5 (Khosravi-Darani et al. 2008) have also been reported for maximum protease production. Incubation temperature is a crucial environmental parameter for the production of proteases, since it affects microbial growth, regulates synthesis and secretion of the enzyme by changing the properties of the cell wall (Ibrahim et al. 2015b). Optimum temperatures of 30 °C, 37 °C, 40 °C, and 60 °C for protease

production by *Pseudomonas aeruginosa* MCM B-327 (Zambare et al. 2011), *Bacillus subtilis* AKRS3 (Ravishankar et al. 2012), *Bacillus* sp. NPST-AK15 (Ibrahim et al. 2015b), and *Bacillus polymyxa* (Maal et al. 2009), respectively have been reported.

Statistical approaches for optimization of protease production

Since microbial extracellular protease production is significantly influenced by media components, optimization of these parameters is imperative in order to maintain a balance between various components for enhanced protease production (Kumar and Takagi 1999; Chandra et al. 2015). Process optimization has become a topic of central importance in industrial production processes with special regard to biotechnology (Reddy et al. 2008). This technique is found suitable for improved productivity of any product of interest. The conventional one-variable-at-a-time (OVAT) approach involving change of one independent variable while maintaining all others at a fixed level is the mostly frequently used technique in biotechnology to obtain maximum cell growth and high yields of desired metabolite. However, this approach is not only time-consuming and costly for a large number of variables, but also eliminates combined interactions among the parameters and often leads to misinterpretation of results when interactions between different components are present (Li et al. 2008). In order to overcome these challenges, statistical experimental designs such as Plackett-Burman design (PBD), Taguchi methodology, response surface methodology (RSM) and artificial neural network model are widely used as powerful tools for optimization processes (Dutta et al. 2004; Oskouie et al. 2007; Chavan et al. 2015) (Table 2).

Plackett-Burman design

Plackett-Burman design is a two-level design used for the selection of significant factors from a huge number of process parameters, and thus useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated in further optimization processes (Saxena and Singh 2010; Singh et al. 2017). In this design, there are two types of variables, namely real variables whose concentration changes during the experiments, and dummy variables whose concentration remains constant during the experiments. Each variable is represented in two levels, high and low. The use of PBD tremendously reduces the total number of experiments, since the interaction effects of the variables are not considered and only those variables that actually affect the production of desired metabolite are screened (Adinarayana and Ellaiah 2002; Singh et al. 2017). PBD is a reliable approach for evaluation of relative importance of various variables or medium composition for a specific output (response) (Singh and Tripathi 2008; Rajeswari et al. 2014). Optimal conditions for maximum extracellular production of alkaline protease by *Bacillus subtilis* SHmIIIa has been investigated using PBD (Chandra et al. 2015).

Table 2 Statistical methods for optimization of protease production

Microorganism	Design	Parameter optimized	Yield improvement	Reference
Bacillus subtilis K-1	Plackett-Burman and RSM	Peptone, moisture content and temperature	1.4-fold	Singh and Bajaj (2016)
Bacillus clausii	RSM	Sucrose, yeast extract and KNO_3	6-fold	Oskouie et al. 2008
Brevibacterium linens DSM 20158	Plackett-Burman and RSM	Soybean meal, wheat bran, $(NH_4)_2SO_4$ and inoculum size	2-fold	Shabbiri et al. 2012
Marinobacter sp. GA CAS9	Plackett-Burman and RSM	NaCl, beef extract, $CuSO_4$ and pH	3.29-fold	Kumar et al. 2014
Bacillus sp. HTS102	RSM	Temperature, peptone, pH and yeast extract	2.3-fold	Queiroga et al. 2012
Bacillus sp.	Plackett-Burman and RSM	Glucose, soybean and K_2HPO_4	2-fold	Saxena and Singh (2010)
Bacillus mojavensis A21	Plackett-Burman and RSM	Chickenpea flour, fababean flour, inoculum size, incubation time and temperature	5-fold	Mhamdi et al. 2014
Bacillus subtilis SHmIIIa	Plackett-Burman	pH, temperature, agitation, inoculum size, glucose, peptone, KH_2PO4, $FeSO_4$, Tween 20	37.26-fold	Chandra et al. 2015
Bacillus clausii	Taguchi	Sucrose, yeast extract, KNO3, trace element, agitation	4-fold	Oskouie et al. 2007
Haloferax lucentensis VKMM 007	RSM	KCl, $MgSO_4$, gelatin and soluble starch	4-fold	Manikandan et al. 2011
Rheinheimera sp	RSM	Temperature, pH, skim milk and inoculum size	8-fold	Mahjoubin-Tehran et al. 2016
Halobacterium sp. SP1 (1)	Plackett-Burman and RSM	Soybean flour and $FeCl_3$	3.9-fold	Akolkar et al. 2009
Aspergillus niger I1	Plackett-Burman and RSM	KH_2PO_4, intial pH and temperature	4.25-fold	Siala et al. 2012
Aspergillus clavatus ES_1	Plackett-Burman and RSM	*Mirabilis jalapa* tuber powder, temperature and pH	14-fold	Hajji et al. 2008

In this study, nine process parameters including pH, temperature, agitation speed, inoculum volume, glucose, peptone, KH_2PO_4, $FeSO_4$ and Tween 20 at two levels were selected for the design. Out of which, only six parameters (pH 9.75, agitation speed 225 rpm, inoculum volume 3.5%, glucose 5.5 g/l, peptone 3.5 g/l and KH_2PO_4) showed significant influence on alkaline protease production. The optimization process led to 37.26-fold increase in protease production.

Response surface methodology

Response surface methodology is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where response of interest is influenced by several parameters with the sole purpose of optimizing the response (Oskouie et al. 2008). RSM employs several phases of optimization, and it can be performed in three basic steps, namely design of experiments for screening of the parameters followed by the path of steepest ascent/descent, and finally quadratic regression model is fitted and optimized using canonical regression analysis method (Gupte and Kulkarni 2003; Singh et al. 2017). This method allows building of models for accurate approximation of true response function within a region around the optimum, using process parameters as independent variables (Puri et al. 2002; Queiroga et al. 2012). RSM is useful in confirming the effects and interactions of fermentation variables. In addition, it results in improved productivity, lessens process changeability, gives closer confirmation of the predicted response to the experimental values and reduces overall costs (Shabbiri et al. 2012). This experimental design is more useful for optimization of industrial processes and has been applied in various fields including biomedicine, food and agriculture (Wang et al. 2017). Several fermentation parameters have been optimized using RSM (Govarthanan et al. 2014; Kandasamy et al. 2016; Singh and Bajaj 2016; Vijayaraghavan et al. 2017). Experimental designs such as central composite design (CCD), Box-Behnken design (BBD) and Doehlert design are widely used in RSM to approximate a response function to experimental data that cannot be described by linear functions (Li et al. 2007).

Central composite design

The CCD is widely employed in RSM for building a second order (quadratic) model for the response variable without using a complete three factorial experiments (Singh et al. 2017). It consists of a full factorial or fractional factorial design, a star design in which experimental points are at a distance of α from its center, and a central point. It has extensively been applied for optimization of protease production (Puri et al. 2002; Oskouie et al. 2008; Shabbiri et al. 2012; Queiroga et al. 2012; Singh and Bajaj 2016). Statistical optimization of medium components and culture conditions for alkaline protease production by *Marinobacter* sp. GA CAS9 using CCD has been reported (Kumar et al. 2014). Results obtained revealed that four independent variables including NaCl (60.53 g/l), beef extract (14.73 g/l), $CuSO_4$ (4.73 g/l) and

pH (10.7) significantly influenced protease production. The experimental protease production was found to correspond with the predicted value, an indication of accuracy of the model. Enhancement of protease production from 298.34 U/ml to 982.68 U/ml was recorded after the optimization process. Saxena and Singh (2010) employed RSM for the optimization of various bioprocess parameters for protease production by *Bacillus* sp. Preliminary screening of bioprocess parameters including glucose, soybean, K_2HPO_4, NaCl, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, agitation, inoculum size, pH and incubation period for maximum protease production was carried out using PBD. Variables (glucose, soybean, K_2HPO_4) that showed significant effects on protease production were further optimized using CCD. Results obtained showed an approximately two-fold increase in protease production using basal medium as reference. Singh and Bajaj (2016) optimized production of thermoalkali-stable protease from *Bacillus subtilis* K-1 using agro-industrial waste as a substrate. Results obtained from PBD showed that peptone, moisture content, temperature, phosphates, and inoculum volume significantly influenced protease production. In addition, statistical optimization of the selected significant variables, namely peptone, moisture content, and incubation temperature by CCD resulted in 40% (from initial 728 U/ml to 1020 U/ml) enhancement in protease production.

Box-Behnken design

Unlike CCD, BBD is independent of quadratic design, and lack an embedded factorial or fractional design (Ferreira et al. 2007). It requires fewer experiments and a shorter cycle time for a multi-variable optimization in a three-level (-1, 0 and +1) system (Wang et al. 2017). It is formed by combination of two-level factorial designs with incomplete block designs. BBD is an attractive, efficient and productive tool used to enhance the production of microbial metabolites (Govarthanan et al. 2014; Kandasamy et al. 2016; Wang et al. 2017). Kandasamy et al. (2016) applied RSM-based BBD for the optimization of variables including pH, incubation time, coffee pulp waste and corncob substrate concentration for enhanced protease production by *Bacillus* sp. Maximum yield (920 U/ml) of protease production was achieved after 60 h of incubation with 3.0 g/l coffee pulp waste, and 2.0 g/l corncob at pH 8.0 and temperature of 37 °C. A 5-fold increase in protease production by *Bacillus mojavensis* A21 following optimization of medium components and culture conditions through BBD has been reported (Mhamdi et al. 2014).

Purification of proteases

Crude proteases are commonly employed for commercial applications, where high enzyme purity is not required. This makes the application of enzymes cost-effective, since purification into pure form is tedious, time-consuming and very expensive (Rigo et al. 2008; Mugdha and Usha 2012). The details of downstream purification techniques are discussed below:

Ultrafiltration

Because of the low amounts of enzyme in the cell-free supernatant, excess water is usually removed for the recovery of the enzyme. This is achieved *via* membrane separation processes such as ultrafiltration. This pressure-driven separation process is inexpensive, leads to a slight loss of enzyme activity and used for purification, concentration and diafiltration or for changing the salt composition (Sullivan et al. 1984; Kumar and Takagi 1999). However, its major drawbacks include fouling or clogging of membrane resulting from precipitates formed by the final product.

Precipitation

Precipitation is the most frequently used technique for the separation of enzymes from crude culture supernatants (Bell et al. 1983). It is carried out by addition of inorganic salt (ammonium sulphate) or organic solvent (acetone or ethanol) which reduces the solubility of the desired enzymes in an aqueous solution (Muthulakshmi et al. 2011).

Ion exchange chromatography

Proteases are positively charged biomolecules, and are not bound to anion exchangers (Kumar and Takagi 1999). As a result, cation exchangers form a rational choice for the elution of the bound molecules from the column by increasing the salt or pH gradient (Gupta et al. 2002). The commonly employed matrices for ion-exchange chromatography include diethyl amino ethyl and carboxy methyl, which upon binding to the charged enzyme molecules adsorb the proteins to the matrices. Elution of the adsorbed protein molecule is achieved by a gradient change in pH or ionic strength of the eluting buffer (Gupta et al. 2002).

Affinity chromatography

The most commonly used adsorbents for protease purification include hydroxyapatite, immobilized N-benzoyloxycarbonyl phenylalanine agarose, immobilized casein glutamic acid, aprotinin-agarose, and casein-agarose. However, the ultimate disadvantage of this technique is the high cost of enzyme supports and the labile nature of some affinity ligands, thus reducing the use at process scale (Kumar and Takagi 1999; Gupta et al. 2002).

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography is based on variation of external hydrophobic amino acid residues on different proteins, resulting in protein interaction. In aqueous solvent, hydrophobic patches on proteins preferentially interrelate with other hydrophobic surfaces. These hydrophobic interactions are reinforced

by high salt concentrations and higher temperature, and are weakened by the presence of detergents or miscible organic solvents. The degree of binding of a hydrophobic protein depends on the type and density of substitution of the matrix, as well as on the nature of buffer conditions (Gupta et al. 2002).

Immobilization of proteases

Enzyme immobilization refers to as physical confinement or localization of enzymes in a certain defined region of space with retention of their catalytic activities (Mohamad et al. 2015). Immobilization of enzyme onto appropriate insoluble support is an important tool to fabricate a range of functional properties (Tischer and Wadekind 1999). Immobilization offers many distinct advantages including possibility of continuous process, rapid termination of reactions, controlled product formation, and ease of enzyme separation from reaction mixture (Datta et al. 2013). In addition, insolubilization of enzyme by attachment to a matrix provides several benefits such as enhanced stability, possible modulation of the catalytic properties, reduction in the cost of enzymes and enzyme products, and adaptability to various engineering designs (Asuri et al. 2006; Sheldon 2007; Tian et al. 2009; Hernandez et al. 2011; Saifuddin et al. 2013).

The characteristics of a matrix are crucial in determining the effectiveness of the immobilized enzyme system (Mohamad et al. 2015). The selection of appropriate support material influences the immobilization process. Ideal support properties include hydrophilicity, inertness towards enzymes, biocompatibility, non-toxicity, biodegradability, resistance to microbial attack, resistance to compression and readily affordable at low cost (Singh 2009). The support materials can be classified as organic and inorganic based on their chemical components, and can further be sub-divided into natural and synthetic polymers. These include carboxymethyl cellulose, starch, collagen, modified sepharose, ion exchange resins, active charcoal, silica (Wu et al. 2012), clay (Kim et al. 2006), aluminium oxide, titanium, diatomaceous earth, hydroxyapatite, ceramics, celite (Khan et al. 2006; Ansari and Husain 2012; Datta et al. 2013), agarose (Gemenier 1992; Brena and Viera 2006), treated porous glass (Gorecka and Jastrzebska 2011) and certain polymers (Lee et al. 2009).

The choice of suitable immobilization technique is paramount for the immobilization process, as it determines the enzyme activity and characteristics in a particular reaction (Mohamad et al. 2015). Methods such as adsorption, entrapment, covalent bonding and cross-linking are commonly used for enzyme immobilization (Huang et al. 2011; Sheldon 2011; Geethanjali and Subash 2013; Park et al. 2013; Sahin et al. 2015). Immobilization of alkaline protease from *Bacillus amyloliquefaciens* SP1 by entrapment in various matrices including alginate, agar and polyacrylamide has been reported (Guleria et al. 2016a). The immobilized enzyme showed enhanced protease activity and reusability with beads prepared with different polymers. In addition, *Bacillus subtilis* M-11 protease immobilized on polysulfone membrane (containing

silica gel-3 aminopropyltriethoxysilane) by physical adsorption displayed improved stability and retention of its activity (77.3%) after ten repeated use (Sahin et al. 2015). Ibrahim et al. (2016) immobilized alkaline protease from *Bacillus* sp. NPST-AK15 onto hollow core-mesoporous shell silica nanospheres by covalent attachment and physical adsorption. The immobilized enzyme recorded significant thermal and organic solvent stability with a considerable catalytic activity for twelve consecutive batches. Silva et al. (2006) studied the immobilization of alkaline protease, Esperase by covelent bonding to Eudragit S-100 through carbodiimide coupling. The immobilized enzyme exhibited a good thermal and storage stability, and reusability in comparison to the native enzyme.

Characterization of alkaline proteases

Alkaline proteases from different microorganisms are well studied for suitability for various specific applications based on their properties (Kumar and Takagi 1999). For biotechnological applications, proteases must possess higher activity and stability under relatively extreme temperature, pH etc. (Sharma et al. 2017). The essential properties of some microbial alkaline proteases are presented in Table 3 and discussed in details below:

Effect of pH on activity and stability

A significant level of proteolytic activity over a broad range of pH is required for protease to be employed for various biotechnological applications (Adinarayana et al. 2003). In general, bacterial alkaline proteases exhibit high activity at optimum pH range of between 8.0 and 12.0 (Jisha et al. 2013). Optimum pH and stability of a serine alkaline protease from *Aeribacillus pallidus* C10 has been reported (Yildirim et al. 2017). The enzyme was found to be active within a broad pH range of 7.0-10.0, with maximum activity recorded at pH 9.0. The protease retained its activity by more than 70% in the range of pH 6.0-10.5 after 2 h incubation. Alkaline proteases from *Bacillus pumilus* CBS (Jaouadi et al. 2008), *Bacillus* strain HUTBS71 (Akel et al. 2009) and *Bacillus licheniformis* (Sarker et al. 2013) with similar pH stability profiles have been reported. Ibrahim et al. (2015a) assessed the influence of pH on the activity and stability of protease produced by *Bacillus* sp. NPST-AK15. The enzyme was active in a wide pH range (7.0-12.0), with maximum activity recorded at pH 10.5. The protease was 100% stable at pH 9.0-10.5, retaining 96.6 and 92.3% of its activity at pH 8.0 and 11.0, respectively, and more than 80% of its initial activity retained at pH 12.0 after 2 h. Protease from *Bacillus circulans* MTCC 7942 exhibited activity in the range of 8.0-13.0 with optimum activity recorded at pH 10.0. The enzyme maintained its stability in a wide range of pH (7.0-12.0) for 24 h, retaining 90% activity in the pH range (8.0-12.0) (Patil et al. 2016).

Table 3 Properties of some microbial alkaline proteases

Microorganism	pH Optima	Temperature optima	Kinetics parameter (K_m and V_{max})	Substrate specificity	Reference
Bacillus sp. CL18	8.0	55 °C	-	Casein and Soy protein	Rieger et al. 2017
Bacillus caseinilyticus	8.0	60 °C	-	Casein, bovine serum albumin, gelatin and egg albumin	Mothe and Sultanpuram 2016
Bacillus licheniformis A10	9.0	70 °C	0.033 mg/ml & 8.17 µmol/ml/min	Casein	Yilmaz et al. 2016
Bacillus licheniformis UV-9	11.0	60 °C	5 mg/ml & 61.58 µM/ml/min	Casein, haemoglobin and bovine albumin	Nadeem et al. 2013
Bacillus pumilus MCAS8	9.0	60 °C	-	Bovine serum albumin, casein, haemoglobin, skim milk, azocasein and gelatin	Jayakumar et al. 2012
Bacillus pseudofirmus	10	50 °C	0.08 mg/ml & 6.346 µM/min	Casein	Raval et al. 2014
Bacillus circulans MTCC 7942	10	60 °C	3.1 mg/ml & 1.8 µmol/min	Casein	Patil et al. 2016
Bacillus circulans M34	11	50 °C	0.96 mg/ml & 9.548 µmol/ml/min	Casein, ovalbumin and bovine serum albumin	Sari et al. 2015
Bacillus amyloliquefaciens SP1	8.0	60 °C	0.125 mg/ml & 12820 µg/ml	Casein	Guleria et al. 2016b
Bacillus sp. NPST-AK15	10.5	60 °C	2.5 mg/ml & 42.5 µM/min/mg	Gelatin, bovine serum albumin and casein,	Ibrahim et al. 2015a
Stenotrophomonas maltophilia SK	9.0	40 °C	-	Bovine serum albumin, casein and gelatin	Waghmare et al. 2015
Stenotrophomonas sp. IIIM-ST045	10.0	15 °C	-	-	Saba et al. 2012
Aeribacillus pallidus C10	9.0	60 °C	0.197 mg/ml & 7.29 µmol/ml/min	Casein	Yildirim et al. 2017
Geobacillus toebii LBT 77	13.0	95 °C	1 mg/ml & 217.5 U/ml	-	Thebti et al. 2016
Streptomyces sp. M30	9.0	80 °C	35.7 mg/ml & 5×10^4 U/mg	Casein, bovine serum albumin, bovine serum fibrin	Xin et al. 2015
Alternaria solani	9.0	50 °C	-	-	Chandrasekaran and Sathiyabama (2014)
Beauveria bassiana AM-118	8.0	35-40 °C	0.216 and 0.7184 mM & 3.33 and 1.17 U/mg	-	Firouzbakht et al. 2015

Similar results have also been reported for proteases from *Bacillus tequilensis* P15 (Bose et al. 2014), *Bacillus subtilis* AP-MSU6 (Maruthiah et al. 2013), *Bacillus circulans* (Benkiar et al. 2013), *Bacillus lehensis* (Joshi and Satyanarayana 2013), and *Bacillus alveayuensis* CAS 5 (Annamalai et al. 2014) showing optimal pH in the range of 8.0-12.0. Maximum activity of a serine protease from *Bacillus pumilus* MCAS8 at pH 9.0 and stability in the range of 7.0-11.0 after 30 min has been observed (Jayakumar et al. 2012). Remarkably, alkaline serine protease from *Bacillus circulans* M34 showed maximum activity at optimum pH of 11.0 and was found to be active over a broad pH ranges (4.0-12.0) (Sari et al. 2014). The enzyme was stable over a wide pH range, maintaining 97% of its original activity at pH 8.0-11.0 after 1 h.

Effect of temperature on activity and stability

Most of the bacterial alkaline proteases are active and stable at a broad range of temperature, from 50 to 70 °C. The activity of alkaline proteases at broad temperatures and thermostability forms a crucial feature required for various biotechnological applications. Alkaline proteases from *Bacillus* sp., *Streptomyces* sp. and *Thermus* sp. are stable at high temperatures; addition of calcium chloride further improves the enzyme thermostability (Nilegaonkar et al. 2006). In addition, some alkaline proteases possess exceptionally high thermostability with no decrease in activity at 60-70 °C for up to 3 h (Adinarayana et al. 2003). Ahmetoglu et al. (2015) investigated the characteristics of protease from *Bacillus* sp. KG5. The enzyme was found to be active at 40-45 °C and stable at 50 °C in the presence of 2 mM $CaCl_2$ after 120 min. Thebti et al. (2016) characterized a haloalkaline protease from *Geobacillus toebii* LBT 77 newly isolated from Tunisian hot spring. The enzyme was active between 70 and 100 °C with an optimum activity recorded at 95 °C. The protease was extremely stable at 90 °C after 180 min. Similar results have also been reported for protease from *Bacillus* sp. MLA64 (Lagzian and Asoodeh 2012). This activation and stability at higher temperature was probably due to the partial thermal inactivation of the protease. Alkaline protease from *Bacillus caseinilyticus* was found to be active at 30-60 °C, with maximum activity attained at 60 °C, indicating thermotolerant nature of the enzyme (Mothe and Sultanpuram 2016). Maximum proteolytic activity of *Bacillus* strains HR-08 and KR-8102 isolated from soil of western and northern parts of Iran has been recorded at 65 and 50 °C, respectively (Moradian et al. 2006). A serine alkaline protease from *Bacillus subtilis* DR8806 showed highest activity at 45 °C and was stable up to 70 °C (Farhadian et al. 2015). Alkaline protease from *Bacillus cohnii* APT5 has been reported to be active at a broad range of temperatures, between 30 and 75 °C with maximum activity attained at 50 °C (Tekin et al. 2012). The enzyme was found to be stable from 40 to 70 °C.

Kinetics properties of alkaline proteases

Since enzymes are natural catalysts that accelerate chemical reactions, the speed of any fastidious reaction being catalyzed by a particular enzyme can only reach a certain maximum value. This is known as maximum velocity (V_{max}) whereas, Michaelis-Menten constant (K_m) is the concentration of substrate at which half of the maximal velocity was attained (Ahmed et al. 2011). The relationship between the rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate. This is usually expressed as the K_m (Ahmed et al. 2011). An enzyme with low K_m has a greater affinity for its substrate. Both K_m and V_{max} are important for developing an enzyme-based process. Knowledge of such parameters is essential for assessing the commercial applications of protease under different conditions (Gupta et al. 2002; Sugumaran et al. 2012). Various substrates including casein, azocasein etc. are employed for determining the kinetic properties of alkaline proteases. Different K_m and V_{max} values have reported for various alkaline proteases. The K_m and V_{max} values of alkaline protease from *Bacillus licheniformis* A10 were determined to be 0.033 mg/ml and 8.17 µmol/ml/min, respectively in the presence of casein (Yilmaz et al. 2016). This K_m value was found to be lower when compared to that of alkaline proteases from *Bacillus licheniformis* UV-9 (Nadeem et al. 2013), *Bacillus circulans* (Rao et al. 2009) and *Bacillus* sp. (Jain et al. 2012), suggesting a high affinity of the enzyme for the substrate. In another study, K_m and V_{max} values of 0.626 mM and 0.0523 mM/min, respectively were recorded for protease from *Bacillus licheniformis* BBRC 100053 using casein (Nejad et al. 2014). Alkaline protease from *Bacillus amyloliquefaciens* SP1showed K_m and V_{max} values of 0.125 mg/ml and 12820 µg/min, respectively in the presence of casein, indicating high affinity and efficient catalytic activity of the enzyme (Guleria et al. 2016b).

Potential applications of alkaline proteases

Alkaline proteases are robust enzymes with significant biotechnological applications in detergents, leather processing, silver recovery, medical, food processing, feeds, and chemical industries, as well as in several bioremediation processes, contributing to the formation of high value-added products (Jisha et al. 2013; Aruna et al. 2014). The various applications of alkaline proteases are discussed below:

Detergent industry

The detergent industry forms the largest industrial application of enzymes, accounting for 25-30% of the total worldwide markets of enzymes and expected to grow faster at a compound annual growth rate of about 11.5% from 2015 to 2020 (Global Market for Enzymes in Industrial Applications 2014). Microbial alkaline proteases are dominant in commercial applications with a substantial share of market utilized in laundry detergent (Kumar and Takagi 1999). They are used as additives in detergent formulations for the removal

of proteinaceous stains resulting from food, blood, and other body secretions, as well as to improve washing performance in domestic laundry and cleaning of contact lenses or dentures (Rao et al. 1998; Grbavčić et al. 2011; Baweja et al. 2016). The use of alkaline proteases in detergent products offers enormous advantages since these products contain less bleaching agents and phosphates. Thus, rendering beneficial effects on public and environmental health (Olsen and Falholt 1998; Novozyme 2013). Generally, an ideal alkaline protease used as detergent additives should have a long shelf life, as well as high activity and stability over a wide range of pH and temperature. In addition, the enzymes should be efficient at low amounts and compatible with various detergent components along with chelating and oxidizing agents (Rao et al. 1998; Kumar and Takagi 1999). This is noteworthy of alkaline proteases from *Bacillus cereus*, *Bacillus pumilus* CBS, *Bacillus licheniformis*, *Bacillus brevis*, and *Bacillus subtilis* AG-1 reported to exhibit robust detergent compatibility in the presence of calcium chloride and glycine used as stabilizers (de Nascimento and Martins 2006; Ghafoor and Hasnain 2009; Abou-Elela et al. 2011; Bezawada et al. 2011; Jaouadi et al. 2011).

Leather industry

Leather processing involves series of stages including curing, soaking, liming, dehairing, bating, picking, degreasing and tanning (Mojsov 2011). Conventional approaches of leather processing involving the use of hazardous chemicals such as sodium sulfide, lime and amines generate severe health hazards and environmental pollution (Choudhary et al. 2004). As a result, the use of biodegradable enzymes as substitutes to chemicals has proved successful in enhancing leather quality and reducing environmental pollution (Rao et al. 1998; Zambare et al. 2011; Adrio and Demain 2014). Enzymatic dehairing processes are attractive in preserving the hair and contribute to fall in organic load discharged into effluent. In addition, it minimizes or eliminates the dependence on toxic chemicals (de Souza and Gutterres 2012). Due to their elastolytic and keratinolytic activity, alkaline proteases are employed for selective breakdown of non-collagenous constituents of the skin and for elimination of non-fibrillar proteins during soaking, in bating, thus producing soft, supple and pliable leather (Singh et al. 2016). Furthermore, microbial alkaline proteases are employed for quick absorption of water thus, reducing soaking time (Jaouadi et al. 2013). Alkaline proteases from *Bacillus* sp. with keratinolytic activity have been reported for dehairing properties (Arunachalam and Sarita 2009; Vijayaraghavan et al. 2014; Kshetri and Ningthoujam 2016; Rieger et al. 2017).

Food industry

In food industry, alkaline proteases are usually used for various purposes including cheesemaking, baking, preparation of soya hydrolysates, meat tenderization etc. The catalytic function of proteases is used in the

preparation of high nutritional value protein hydrolysate, used as components of dietetic and health products; in infant formulae and clinical nutritional supplements, and as flavoring agents (Gupta et al. 2002; Ward 2011). However, the bitter taste of protein hydrolysate formed a crucial barrier for use in food and health care products. Therefore, proteases (carboxypeptidases A) have a high specificity for debittering protein hydrolysates. A key application of protease in dairy industry is in the cheese manufacturing, where the primary role of the enzymes is to hydrolyze specific peptide to generate casein and macropeptides (Rao et al. 1998; Pai 2003; Qureshi et al. 2015). In addition, proteases play a significant role in meat tenderization, particularly of beef, since they possess the potential to hydrolyze connective tissue proteins as well as muscle fiber proteins (Kumar and Takagi 1999). Endo- and exoproteinases are used in baking industry to modify wheat gluten. The addition of bacterial proteases reduces the mixing time, improves extensibility and strength of dough and results in enhanced loaf volume (Rao et al. 1998). Alkaline proteases are employed in processing of soy sauce and soy products. Proteases are also used in the enzymatic synthesis of aspartame (sweetening agent).

Medicine

A broad diversity and specificity of proteases are employed for the development of various therapeutic agents. For instance, elastoterase from *Bacillus subtilis* 316M immobilized on a bandage has been used for the treatment of burns, purulent wounds, carbuncles, furuncles, and deep abscesses (Kudrya and Simonenko 1994). In addition, alkaline-fibrinolytic protease has been used as a thrombolytic agent (Kim et al. 1996). Serratiopeptidase, a protease produced by *Serratia* sp. is the most effective protease for treatment of acute and chronic inflammation (Vaisar et al. 2007). Collagenases with alkaline protease activity are used for the preparation of slow-release dosage forms as well as in wound healing, treatment of sciatica in herniated intervertebral discs, treatment of retained placenta, and as a pretreatment for enhancing adenovirus-mediated cancer gene therapy (Watanabe 2004). The cytotoxic nature of several proteases allows the enzymes to be used as efficient antimicrobial agents for clinical purposes (Siritapetawee et al. 2012).

Waste treatment

Alkaline proteases are employed in the treatment of wastes from various food processing industries and household activities. These enzymes solubilize proteinaceous wastes *via* a multistep process for the recovery of liquid concentrates or dry solids of nutritional value for fish or livestock (Karam and Nicell 1997; Karigar and Rao 2011; Pandey et al. 2011). This is achieved by initial adsorption of the enzyme on the solid substrate followed by cleavage of polypeptide chain that are loosely bound to the surface. Thereafter, the solubilization of the more compact core occurs at a slower rate depending on the diffusion of the enzyme surface active sites and core particles (Venugopal et al. 1989). Enzymatic degradation of

wastes using alkaline proteases with keratinolytic activity is an attractive method. Among microbial sp., some members of the genus *Bacillus* are reported as keratinase producers for feather degradation (Kojima et al. 2006; Cortezi et al. 2008; Ni et al. 2011). Enzymatic treatment of waste feather from poultry slaughterhouses using alkaline protease produced by *Bacillus subtilis* has been reported (Dalev 1994). Pretreatment with NaOH, mechanical disintegration, and enzymatic hydrolysis resulted in complete solubilization of the feathers, releasing a heavy, grayish powder with high protein content which could be used as additive in feeds, fertilizers etc. In addition, alkaline proteases with keratinolytic activity are used for degradation of waste material in household refuse, and as a depilatory agent for the removal of hair in bathtub drains, which caused unpleasant odors (Mukhopadhyay and Chandra 1992; Takami et al. 1992).

Conclusion and future perspectives

Proteases represent one of the largest groups of industrial enzymes, constituting more than 65% of the total worldwide sale of enzymes. They are ubiquitous in nature, found in a wide array of plants, animals, and microorganisms. However, microbial proteases are recognized as an excellent source of enzymes due to their broad biochemical diversity, rapid growth, limited space requirement for cultivation, and susceptibility to genetic manipulation. Microbial alkaline proteases play a vital role in several industries, mainly food, detergent, leather and pharmaceutical industries as well as in bioremediation processes. In view of the growing and diverse potential applications of these proteases, further discovery and engineering of novel enzymes with robust catalytic activity will be ultimate developments in the near future through newer methods such as *in vitro* evolutionary changes of protein primary structures.

Conflict of interest

The authors declare that there is no conflict of interest in the publication of this article

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CHAPTER FOUR

PRODUCTION, PROPERTIES AND BIOTECHNOLOGICAL APPLICATIONS OF MICROBIAL LIPASES -- A REVIEW

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Production, properties and biotechnological applications of microbial lipases -- A Review

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Lipases are a class of enzymes that catalyze the hydrolysis and synthesis of esters that formed from glycerol and long-chain fatty acids in oil-water interface, as well as catalyze reverse reactions of esterification, inter-esterification and trans-esterification in nonaqueous and microaqueous milieu. They are widely distributed in nature, and microbial lipases are recognized as excellent source due to their stability, substrate specificity, lower production costs and ease of genetic manipulation. Alkaline lipases from microbial origin have gained intensified attention for a wide range of applications in food, detergent, and cosmetics industries as well as in environmental bioremediation. This review provides insight on different sources and types of lipases, with special emphasis on microbial lipases. Various methods for detection of lipase production and factors influencing its synthesis were further elucidated. In addition, the review describes the use of statistical experimental designs as an efficient tool for optimization of lipase production; different techniques for lipase purification, and immobilization of lipase on a range of support materials. Catalytic characteristics of the enzyme were further reported with emphasis on the activity, stability and kinetics properties of alkaline lipases for possible exploitation in various biotechnological applications.

Keywords: Lipases, Alkaline lipases, Production, Characterization, Industrial applications

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a class of enzymes that catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol and free fatty acids at the organic-aqueous interface. In addition, they catalyze a plethora of reactions including esterification, inter-esterification, trans-esterification, alcoholysis, acidolysis, and aminolysis in non-aqueous and micro-aqueous milieu [4]. Lipases represent the third most commercialized enzymes, after proteases and carbohydrases, and account for more than one-fifth of the global enzyme market [2, 3]. They are naturally produced by plants, animals and microorganisms. However, microbial lipases have attracted special industrial attention owing to their

stability, selectivity and broad substrate specificity [20]. Lipases are serine hydrolases and their activity rely on a catalytic triad consisting of Ser-Asp/Glu-His with a consensus sequence (Gly-x-Ser-x-Gly) [5, 6]. The three-dimensional structures of lipases reveal the characteristic *α/β* hydrolase fold [7]. The active site of the *α/β* hydrolase fold enzymes consists of three catalytic residues namely, nucleophilic residue, catalytic acid residue, and histidine residue [8]. Lipases exhibit chemo-specificity, regio-selectivity and enantio-selectivity toward substrates [9, 10, 11, 12]. Lipases have emerged as one of the leading biocatalysts with proven potential biotechnological applications, contributing to the multi-billion dollar under-exploited lipid technology-based bio-industry [1]. They are employed in various industries ranging from biodiesels, food, nutraceuticals, detergents, bioremediation, agriculture, cosmetics, leather and paper industries [1]. The present review aims at providing insight on microbial lipases, with special focus on their production and properties for exploitation for various biotechnological applications.

2. Sources of lipases

Lipases are ubiquitous in nature and are produced by various plants, animals and microorganisms.

2.1. Plant lipases

In plants, true lipases are abundant in oil bodies of oleaginous seeds including sunflower seeds, linseed, and bean seeds as well as in cereals [12, 13, 14]. The lipolytic enzymes in the seeds are required for the mobilization of stored fatty acids, needed as sole carbon and energy source for seedling growth [15]. A putative lipase involving in the mobilization of fatty acids in the plastoglobuli of chloroplasts has also been discovered [16]. Latex, a milk sap of rubber trees has been reported to contain lipase activity [17]. In addition, *Carica papaya* lipase has emerged as a versatile biocatalyst for many biotechnological applications including modification of fats and oils, esterification, inter-esterification etc.

2.2. Animal lipases

These include pancreatic and pregastric lipases, obtained from calf, goat and lamb gullet. They are employed in various industrial processes such as acceleration of cheese ripening and flavor generation, in addition to lipolysis of butter, fat and cream [18]. Lipase of pancreatic juice source also catalyzed the hydrolysis of insoluble oil droplets into soluble products.

2.3. Microbial lipases

Lipases of microbial origin represent the most widely used class of enzymes in biotechnological applications. This is due to their stability at broad ranges of temperature and pH, substrate specificity, high yields, lower production costs, and ease of genetic manipulation [19, 2, 20, 21]. Many microorganisms

including bacteria, fungi, yeasts, and actinomycetes produce lipases [22]. There are various reports available on the production of bacterial lipases, particularly from members of the genera *Acinetobacter* [23, 24, 25], *Bacillus* [26, 27], *Burkholderia* [28], *Pseudomonas* [29], *Staphylococcus* [30], *Microbacterium* [31], *Lactobacillus* [32], *Serratia* [33], *Aeromonas* [34], *Arthrobacter* [35], *Stenotrophomonas* [36], *Thermosyntropha* [37] etc. However, among bacterial genera, *Bacillus* and *Pseudomonas* are the most prominent lipase producers [21]. The lipolytic activity of various fungal and yeast strains has been extensively explored over the last few years. Of the fungal genera, lipases from *Aspergillus* [38], *Mucor* [39], *Penicillium* [40], *Rhizopus* [41], *Fusarium* [42], *Geotrichum* [43], *Antrodia* [44] and *Metarhizium* [45] have been reported. Yeasts have been adequately studied for their lipolytic activity, with *Yarrowia* [46] being the most significant one. Lipolytic activities in *Pseudozyma* [47], *Cryptococcus* [48], *Pichia* [49] and *Rhodotorula* [50] have also been reported. These lipase-producing microorganisms are found in diverse habitats including industrial wastes, vegetable oil processing factories, dairies, oil-contaminated soil, decaying foods, compost heaps, coal tips, marine sponges and hot spring [51, 52, 53]. A list of lipase-producing microorganisms and their sources of isolation is presented in Table 1.

Table 1: Some lipase-producing microorganisms and their sources

Microorganism	Source of isolation	Reference
Bacteria		
Bacillus sp.	Oil-contaminated soil	[63]
Bacillus coagulans	Soil from olive oil processing factory	[184]
Bacillus sp. L2	Hot spring, Perak, Malaysia	[139]
Bacillus sp. FH5	Tannery waste	[148]
Bacillus coagulans BTS-3	Kitchen waste	[185]
Bacillus thermoleovorans CCR11	"El Carrizal" hot springs, Veracruz, Mexico	[66]
Bacillus pumilus RK31	Oil-contaminated soil	[186]
Acinetobacter sp. AU07	Distillery unit	[78]
Acinetobacter sp.	Oil-contaminated soil, South Korea	[68]
Acinetobacter haemolyticus TA 106	Human skin	[187]
Acinetobacter haemolyticus NSO2-30	Olive pomace-soil mixture	[60]
Enterobacter aerogenes IABR-0785	Soil of IIT, Kharagpur	[188]
Burkholderia sp. HL-10	Lipid-contaminated soil	[102]
Geobacillus thermoleovorans YN	Desert soil sample	[189]
*Geobacillus s*p. ARM	Oil-contaminated soil, Selangor, Malaysia	[190]
Geobacillus zalihae	Palm oil effluent, Semenyih, Malaysia	[64]

Pseudomonas sp. BUP6	Rumen of Malabari goat	[191]
Pseudomonas fluorescens RB02-3	Pasteurized and raw milk	[59]
Pseudomonas aeruginosa KM110	Oil processing plant wastewater, Tehran, Iran	[29]
Microbacterium sp.	Pulp and paper mill effluent	[192]
Staphylococcus aureus NK-LB37	Oil-contaminated soil, Coimbatore, Tamilnadu	[193]
Lactobacillus plantarum DSMZ 12028	Dry fermented sausage	[194]
Aeromonas sp. S1	Soil and sludge in oil and grease chamber of dairy industry, New Delhi, India	[195]
Arthrobacter sp. BGCC#490	Oil-contaminated soil of automobile garage	[196]
Stenotrophomonas maltophilia	Soil sample	[36]
Thermomyces lanuginosus	Zoo waste and bird nest materials	[197]
Fungi		
Aspergillus niger DAOM	Dairy effluent	[198]
Aspergillus tamarii JGIF06	Rhizospheric soil, Bangalore, India	[199]
Aspergillus terreus NCFT 4269.10	-	[108]
Trametes hirsuta	Chicken slaughterhouse effluent	[200]
Hypocrea pseudokoningii	Soil samples	[201]
Geotrichum candidum	Soil sample	[202]
Fusarium sp. (*Gibberella fujikuroi* complex)	Decay plant matter in the atlantic forest, São Paulo, Brazil	[203]
Penicillium sp. section *Gracilenta* CBMAI 1583	Atlantic rainforest soil	[204]
Mucor geophillus	Soil sample	[205]
Rhizopus chinensis CCTCC M201021	Da Qu (Traditional leaven for production of Chinese liquor)	[206]
Yeast		
Candida viswanathii	-	[112]
Candida gulliermondi	Leaves of castor bean plant	[207]
Rhodotorula mucilaginosa MTCC 8737	Marine soil sample, Mangalore, India	[50]
Aureobasidium pullulans HN2.3	Sea saltern, Qingdao	[208]
Cryptococcus sp. MTCC 5455	Air	[209]
Trichosporon coremiiforme	Traditional tannery, Fez, Morocco	[210]

3. Classification of bacterial lipases

Bacterial lipases are classified into eight families; the largest family is sub-divided into six sub-families [54]. This classification is based on conserved sequence motifs and biocatalytic properties of the enzymes. True lipases belong to family I, which comprises of *Pseudomonas*, *Bacillus* and *Staphylococcus* lipases. These lipases possess the conventional catalytic pentapeptide Gly-Xaa-Ser-Xaa-Gly. They are divided into seven sub-families (I.1-I.7) based on structural features, type of secretion mechanism and requirement for lipase-specific foldases, and relationship to other enzyme families [54, 11]. Family II lipases are characterized with Gly-Asp-Ser-Leu motif at the active site, and include esterases of *Streptomyces*, *Aeromonas* and *Salmonella*. Family III contains lipases of *Streptomyces* sp., but in contrast to family II esterase, they are extracellular lipases. Lipases that are similar to mammalian hormone sensitive lipases are grouped under family IV whereas, lipases of mesophilic bacteria including *Pseudomonas oleovorans* and *Haemophilus influenza* belong to family V. Family VI lipases are the smallest esterases, the active enzymes are dimeric. Family VIII lipases are large esterases, consisting of amino acid sequence homologous to that of eukaryotic acetyl choline esterases. Family VIII lipases are similar to β-lactamases. The sequences of few other lipases that are not grouped into any of the eight super-families are arbitrarily classified as new family IX and X. This is typical of a cold active lipase reported by De Pascale et al. [6], and grouped to a novel lipolytic family. However, this classification by Arpigny and Jaeger [54] has been revised several times. Currently, there are XVI families of lipases available in ESTHER database (http://bioweb.ensam.inra.fr/esther). In addition, a new classification recently reported in the lipase engineering database (http://www.led.uni-stuttgart.de) grouped lipases into three classes based on oxyanion hole: GX, GGGX, and Y.

4. Methods for detection of lipase production

Several techniques have been developed for the screening of microorganisms for lipase production. These methods either directly use the microorganism under study or measure lipolytic activity in the crude or purified enzyme preparations [55, 56]. Numerous methods employed for measuring hydrolytic activity and detection of lipase production are discussed below in details:

4.1. Screening methods on solid media

Lipolysis is monitored directly by changes in the appearance of the substrate (such as tributyrin and triolein) that are emulsified in the various growth media [57]. The formation of clear halos around the colonies cultivated on the agar plate is an indication of lipase production [58]. *Pseudomonas fluorescens* RB02-3 and *Acinetobacter haemolyticus* NS02-30 were screened for lipolytic activity on tributyrin agar [59, 60].

Lipolytic *Bacillus* sp. LBN 4 has been isolated on tributyrin agar medium using glycerol tributyrate as substrate [61]. In addition, solid media supplemented with dyes such as phenol red, Victoria Blue B, Spirit blue, or Nile blue sulfate as pH indicator are also used for determination of lipolytic activity [62]. The drop in pH due to the release of fatty acid is indicated by a change in the color of the indicators. This chromogenic method is simple and rapid. However, acidification of the medium resulting from the production of free fatty acids from microbial lipases gives false results [57]. Phenol red agar, consisting of phenol red dye (0.01%, w/v), olive oil (0.1%, v/v), $CaCl_2$ (0.1%, w/v) and Agar (2%, w/v) has been used for screening of *Bacillus* strain [63]. *Geobacillus zalihae* sp. nov. was screened for lipolytic activity using triolein agar plate, comprising of triolein (0.25%, v/v), agar (1%, w/v), nutrient broth (0.8%, w/v) and Victoria Blue (0.01%, w/v) [64]. Furthermore, fluorescent dye Rhodomine B is also employed for detection of lipolytic organisms in plate assay containing emulsified olive oil as substrate. The formation of orange fluorescent halos around colonies under UV irradiation and quantitation of lipase activities range (1 to 30 nkat) suggest production of lipase [65]. Castro-Ochoa et al. [66] screened *Bacillus thermoleovorans* CCR11 for lipolytic activity on Rhodomine B agar consisting of Rhodomine B (0.001%, w/v), nutrient broth (0.8%, w/v), NaCl (0.4%, w/v), olive oil (3%, v/v), and agar (1%, w/v). Spirit blue agar medium has also been used for the detection of lipolytic activity of *Serratia rubidaea* [67] and *Acinetobacter* sp. [68].

4.2. Titrimetric methods

In this technique, lipase activity is measured on a mechanically stirred emulsion of natural or synthetic triacylglycerides by neutralization of free fatty acids released with time following addition of titrated NaOH in order to maintain the pH at a constant end point value [69, 57]. Several authors have reported the use of olive oil as a substrate for the titrimetric analysis [70, 71]. Rasmey et al. [72] measured the lipolytic activity of *Pseudomonas monteilli* 2403-KY120354 in a reaction mixture containing olive oil emulsion incubated at 37 °C for 1 h. Enzyme activity was terminated following addition of 20 ml acetone: ethanol mixture (1:1). The liberated free fatty acids was titrated against 0.1 M NaOH using phenolphthalein. 1 unit of lipase was defined as the amount of enzyme that liberated 1 µmol/min of fatty acids under standard assay conditions. The other least commonly employed techniques for detection of lipase production include chromatographic, turbidimetric, fluorimetric, and immunological methods as well as radioactive assays [57].

5. Lipase production and influencing parameters

Microbial lipases are produced extracellularly by submerged or solid state fermentation during late exponential or stationary phase [51, 73, 74]. However, submerged fermentation involving the growth of microorganisms as a suspension in nutrient enriched liquid medium is mostly preferred due to easily

engineered process control and significant amounts of extracellular enzyme released in the production medium [5, 26]. Lipase production is greatly influenced by nutritional and physicochemical factors, such as temperature, pH, carbon and nitrogen sources, inoculum volume, presence of lipids, inorganic salts, dissolved oxygen concentration, incubation period, and agitation [5, 53]. However, lipidic carbon sources represent the ultimate factor that stimulate high lipase production, since the enzymes are inducible in nature and are therefore generally produced in the presence of a lipid source including oil or other inducers, such as triacylglycerols, Tweens, hydrolysable esters, fatty acids, bile salts, glycerol etc. [51, 5, 75, 76]. For instance, lipase production by *Bacillus flexus* XJU-1 was stimulated by the presence of a surfactant, Tween-80, which favored the uptake of medium components and lipase release [77]. In addition, various oils such as olive oil [27], coconut oil [23], cotton seed oil [77] castor oil [78], soybean oil [30] and neem oil [79] are used as inducers for lipase production. Lipases are produced with a low oil concentration (1-5%) [220, 30]. When a significant amount of oil is used, lipase synthesis reduces due to limitation of oxygen transfer that result in poor microbial growth [77]. Other carbon sources including sugars, sugar alcohol, polysaccharides, whey, casamino acids and other complexes sources influence lipase production [80, 81]. Mannitol was found as the best carbon source for lipase production by *Streptomyces griseochromogenes* [82]. In some cases, combination of carbohydrate and oil is used for maximum lipase secretion [83, 84, 85, 26]. Various non-conventional carbon sources, including beef tallow, wool scour effluent, whey and *n*-hexadecane, as well as cheap agro-industrial residues are also used as lipidic carbon source for lipase production [85, 86, 87].

The type of nitrogen source in the fermentation medium influences the levels of lipase yield by microorganisms [88]. Organic nitrogen sources including peptone, yeast extract or a combination of these resulted in a significant lipase production by most bacterial strains [220, 29, 27]. Optimum lipase production was also recorded with soybean meal [26] and corn steep liquor [28, 24, 25]. The preference of organic nitrogen sources by some lipase-producing microorganisms can be attributed to the presence of some minerals, vitamins or other growth factors that they contain [77]. In some cases, individual amino acids have been reported to play a significant role in lipase production [88]. This is typical of phenylalanine found as a preferred nitrogen source for lipase production by *Streptomyces griseochromogenes* [82]. Inorganic nitrogen sources such as ammonium chloride, ammonium molybdate and diammonium hydrogen phosphate are effective for maximum lipase production [89, 90, 91]. However, a higher nitrogen source concentration inhibits lipase production due to nitrogen metabolite repression [92].

6. Optimization of lipase production using statistical experimental designs

The improvement of industrial fermentation processes is centered on designing of fermentation medium, since its composition considerably influences the yield, concentration and productivity of desired metabolites [5]. The classical optimization by one variable at a time (OVAT) approach is not only time-consuming, laborious and expensive, but also fails to depict the interaction effects of the different variables tested, leading to misinterpretation of results [93]. In order to overcome these difficulties, statistical experimental designs have been recognized as a preferred method for optimization processes [94, 95, 78] (Table 2). The significant variables influencing lipase production are usually screened through the Plackett-Burman design (PBD), and the optimal conditions and interaction effects of these variables are inferred from the response surface methodology (RSM) using Box-Behnken design (BBD) or central composite design (CCD) [21, 96, 97].

Plackett-Burman design has been applied for evaluation of medium components for lipase production through submerged fermentation of microorganisms [98, 99]. PBD, in combination with RSM, has resulted in enhanced feasibility of process scale-up and commercialization of lipase production from a multitude of bacteria, fungi, and yeasts [21]. Ruchi et al. [100] screened eleven media components (peptone, tryptone, NH_4Cl, $NaNO_3$, yeast extract, glucose, glycerol, xylose, gum arabic, $MgSO_4$, and NaCl) for lipase production by *Pseudomonas aeruginosa* using PBD. The most significant parameters (gum arabic, $MgSO_4$, tryptone, and yeast extract) were further optimized by RSM. Maximum lipase yield (5.58-fold) was recorded when tryptone, gum arabic, $MgSO_4$ and yeast extract were at concentrations of 1.01%, 0.02%, 0.10% and 0.02%, respectively. Similarly, the influence of ten medium components (peptone, glucose, NaCl, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CaCl_2$, olive oil, KH_2PO_4, NH_4Cl and Na_2HPO_4) on lipase production by *Alkalibacillus salilacus* SR-079 Halo was studied using PBD [101]. Lipase production was maximally affected by olive oil, KH_2PO_4, NaCl, and glucose. Further optimization of the selected variables by RSM resulted in 4.9-fold enhancement in lipase production at optimal levels of glucose (1g/L), NaCl (4.18 mol/L), olive oil (2%) and KH_2PO_4 (5 g/L).

Combination of OVAT and RSM has been employed for optimization of lipase production. Papagora et al. [94] optimized lipase production from *Debaryomyces hansenii* YLL29 using RSM. The simple one-factor-at-a-time strategy showed that glucose, olive oil and pH were the significant variables influencing lipase production. Further optimization of the selected variables by RSM led to a 2.28-fold increase in lipase production at respective optimal levels of glucose (13.1 g/L), olive oil (19 g/L) and pH (6.4). Similarly, Lo et al. [102] employed RSM and OVAT for the optimization of extracellular lipase production by *Burkholderia* sp. HL-10.

Table 2: Statistical methods for optimization of lipase production

Microorganism	Design	Parameter optimized	Improvement yield	Reference
Debaryomyces hansenii YLL29	RSM	Glucose, olive oil and pH	2.28-fold	[94]
Aspergillus niger G783	RSM	Corn starch, soybean meal and soybean oil	16.4%	[96]
Burkholderia cepacia	RSM	Glucose, palm oil, incubation time, inoculum density and agitation	4-fold	[211]
Enterobacter aerogenes IABR-0785	RSM	Temperature, oil concentration, inoculum volume, pH and incubation period	1.4-fold	[188]
Geobacillus thermoleovorans YN	RSM	Tween 80, olive oil, temperature and pH	4-fold	[189]
Burkholderia sp. HL-10	RSM	Olive oil, tryptone and Tween 80	3-fold	[102]
Geobacillus sp. ARM	RSM and ANN	Temperature, medium volume, inoculum size, agitation rate, incubation period and pH	4.7-fold	[190]
Alkalibacillus salilacus SR-079 Halo	PBD and RSM	Olive oil, KH_2PO_4, NaCl, and glucose	4.9-fold	[101]
Thalassospira permensis M35-15	PBD and RSM	Glucose, peptone, yeast powder and olive oil emulsifier	1.85-fold	[97]
Pseudomonas aeruginosa	PBD and RSM	Gum arabic, $MgSO_4$, tryptone, and yeast extract	5.58-fold	[100]
Candida rugosa NCIM 3462	PBD and RSM	Glucose, groundnut oil, peptone, $(NH_4)_2SO_4$ and $FeCl_3.6H_2O$	1.64-fold	[212]
Fusarium solani SKWF7	RSM	Palm oil, $(NH_4)_2SO_4$ and $CaCO_3$	1.7-fold	[213]
Fusarium verticillioides	RSM	KH_2PO_4, $MgSO_4$, peptone and sunflower oil	2-fold	[214]

Preliminary studies by OVAT revealed that olive oil, tryptone and Tween-80 exhibited significant effects on lipase production. Optimization by CCD resulted in almost 3-fold increase in maximum lipase production at respective optimum concentrations of olive oil (0.65%, v/v), tryptone (2.42%, w/v) and Tween-80 (0.15%, v/v).

7. Purification of lipases

Most of the commercial applications of enzymes do not always require homogenous preparation of the enzyme. However, a certain degree of purity is needed, depending upon the final application in industries such as fine chemicals, pharmaceuticals and cosmetics [103, 5]. For industrial purposes, the purification techniques employed should be cost-effective, rapid, high yielding and amenable to large-scale operations. In addition, they should possess potential for continuous product recovery, with a relatively high capacity and selectivity for the desired product [5]. The extent of purification varies with the order of purification steps.

Most of the microbial lipases are extracellular in nature and the fermentation process is usually followed by the recovery of the biomass from the culture broth by centrifugation or filtration. The obtained cell-free culture supernatant containing the crude enzyme is then concentrated by ultrafiltration, ammonium sulphate precipitation or extraction with organic solvents [221]. Approximately 80% of all the purification methods employed thus far used a precipitation step, with 60% of these using ammonium sulphate and 35% using ethanol, acetone or an acid (usually hydrochloric acid). The precipitation step is normally carried out in the early stages of recovery, followed by chromatographic separation. However, precipitation techniques normally give high yield (87%) in comparison to chromatography methods, which result in lower yields of about 60-70% [104, 221]. Such enzyme preparations are suitable for use in detergent formulations and wastewater treatment [5, 105, 106]. Partial purification of lipases by ultrafiltration [107], ammonium sulphate [108], ethanol [109] or a combination of the precipitating agents has been reported [90]. However, ammonium sulphate is mostly preferred because of its cost-effectiveness, high solubility in water and exhibit no effect on enzyme structure [110]. Increase in lipase activity depends on the concentration of ammonium sulphate used [111].

In industrial applications, such as synthetic reactions in pharmaceutical industry, high purity of lipase is required. Hence, chromatographic techniques involving ion exchange chromatography [26], gel filtration chromatography [60], affinity chromatography [107], hydrophobic interaction chromatography [112] or a combination [28] are mostly used. However, these routine procedures of lipase purification are sometimes time-consuming and result in low final yields. As a result, novel technologies including membrane processes, immunopurification, hydrophobic interaction chromatography using epoxy-activated spacer arm

as a ligand and poly-ethylene glycol-sepharose gel, poly (vinyl alcohol) polymers as column chromatography stationary phases, and aqueous two-phase systems have recently been applied for purification of lipase [221].

8. Immobilization of lipases

Immobilization of lipase on/in a variety of support materials permits multiple reuse of the enzyme, enhanced stability, and easier product recovery [21, 113]. In addition, in comparison to free enzymes, immobilized lipases allow possible continuous process, rapid termination of reactions, and controlled product formation [114, 115]. Such immobilized enzymes are used in a wide range of applications [116, 117]. Methods such as adsorption [118], covalent bonding [119], entrapment [120], and cross-linking [121] are employed for lipase immobilization. For instance, Dandavate et al. [122] covalently immobilized *Candida rugosa* lipase onto the surface of silica nanoparticles and glutaraldehyde-activated aminopropyl glass beads, resulting in easy recovery and reuse of the enzyme. Immobilization of *Yarrowia lipolytica* lipase on octyl-agarose and octadecyl-sepabeads by physical adsorption led to higher yields and greater stability [123]. Among fungi, immobilization of *Rhizopus oryzae* lipase on silica aerogels [124], and *Thermomyces* sp. lipase on regenerated cellulose, glass fiber and polyvinylidene fluoride grafted with 1,4-diaminobutane and activated with glutaraldehyde [125] have been reported. Among bacterial lipases, *Pseudomonas* sp. lipases have been covalently immobilized on porous polymethyl-acrylamide cross-linked with N,N'methylene bisacrylamide [126] and bacterially produced exopolysaccharide [127]. In addition, zirconia particles [85], alginate-k-carrageenan hybrid matrix [128], celite carriers [129], polyglutaraldehyde activated styrene-divinylbenzene copolymer [130], synthetic macroporous alkylated glycidyl epoxy copolymers [131] are notable support materials reported for immobilization of lipases from bacteria including *Burkholderia* sp. and *Arthrobacter* sp.

9. Characterization of alkaline lipases

Lipases are characterized by determining their pH activity and stability, temperature activity and stability, kinetic parameters, among others in order to discover new commercial enzymes with better properties suitable for various biotechnological applications. The essential properties of some microbial alkaline lipases are highlighted in Table 3 and discussed in details below:

9.1. Effect of temperature on activity and stability

Alkaline lipases exhibit optimum activity and stability at broad temperature ranges, a characteristic for adaptability to various industrial applications. Generally, bacterial lipases are active in the temperature range of 30-60 °C [132].

Table 3: Biochemical properties of some microbial alkaline lipases

Microorganism	Optimal pH	Optimal temperature	Stable pH	Stable temperature	Kinetic parameter (K_m and V_{max})	Reference
Acinetobacter sp. EH28	10.0	50 °C	-	-	-	[215]
Acinetobacter haemolyticus NS02-30	9.0	40 °C	5.0-11.0	10-30 °C	0.8 mM & 3.833 mmol/ml/min	[60]
Acinetobacter calcoaceticus 1-7	9.0	40 °C	4.0-10.0	20-50 °C	-	[25]
Acinetobacter sp. AU07	8.0	50 °C	5.0-11.0	40-50 °C	(0.51, 0.15 and 0.17 mM) & (16.98, 15.44 and 14.61 U/mg)	[78]
Pseudomonas aeruginosa BN-1	8.0	37 °C	9.5	50 °C	-	[216]
Bacillus coagulans BTS-3	8.5	55 °C	8.0-10.5	60-70 °C	-	[185]
Bacillus SP. FH5	9.0	60 °C	-	-	0.345 Mm & 7.61 µM/ml/min	[148]
Bacillus sp. L2	9.0	70 °C	8.0-10.0	50 °C	-	[139]
Bacillus smithii BTMS 11	8.0	50 °C	7.0-10.0	30-80 °C	-	[26]
Halobacillus sp. AP-MSU 8	9.0	40 °C	-	-	-	[217]
Geobacillus stearothermophilus AH22	8.0-9.0	50 °C	4.0-10.0	50-60 °C	(0.16, 0.02, 0.19 and 0.55 mM) & (0.51, 1.03, 0.72 and 0.15 U/mg)	[145]
Anoxybacillus flavithermus HBB 134	9.0	50 °C	6.0-11.0	25-50 °C	0.084 mM & 500 U/mg	[146]
Yersinia enterocolitica KM1	9.0	37 °C	7.2-10.0	15-45 °C	16.58 mM & 5.24×10^5 µM/min	[218]
Microbacterium luteolum	8.0	5 °C	5.0-9.0	5-35 °C	-	[31]
Talaromyces thermophilus	9.5	50 °C	9.0-11.0	35-60 °C	-	[87]
Aspergillus japonicus LAB01	8.5	45 °C	5.0-9.0	45 °C	0.13 mM & 12.58 µM/min	[219]
Antrodia cinnamomea BCRC 35396	8.0	45 °C	7.0-10.0	25-60 °C	-	[44]

However, there are reports on lipases that are optimal at both lower and higher temperature ranges [3]. Optimum temperatures of 30, 40, 50, and 60 °C have been reported for lipases produced by *Staphylococcus arlettae* JPBW-1 [30], *Acinetobacter calcoaceticus* 1-7 [25], *Bacillus smithii* BTMS11 [26], and *Bacillus licheniformis* B42 [90], respectively. An extracellular lipase from *Pseudomonas* sp. has been reported to exhibit optimal activity at 90 °C with half-life of more than 13 h at this temperature [133]. Among thermophilic bacteria, lipases from *Bacillus* and *Geobacillus* sp. have been reported to be active at temperature between 50 and 70 °C [134, 66, 107]. Of particular interest are lipases from *Geobacillus kaue* and *Geobacillus kaustophilus* that show optimal activities at 80 °C, which was almost completely maintained at 90 °C [135]. Similarly, thermotolerant lipases, Lip A and Lip B from *Thermosyntropha lipolytica* exhibit maximum activity at around 96 °C, a temperature found to be highest for maximum activity among lipases [136].

Lipases from many microbial species have been reported to be stable at a broad temperature range of between 4 and 90 °C [92]. This is notable of thermostable lipase from a *Bacillus* strain, found to retain 100% of its original activity at 75 °C after 30 min. The enzyme retained 90% of the original activity after being incubated at 60 °C for 15 h [137]. Similarly, a retention of 90% activity at 20-50 °C for 60 h was reported for lipase from *Acinetobacter calcoaceticus* 1-7 [25]. At 30-80 °C, more than 50% activity of lipase from *Bacillus smithii* BTMS 11 was retained after 12 h [26]. Lipase from *Geobacillus thermodenitrificans* IBRL-nra exhibited no loss of activity at 65 and 70 °C after 60 min. However, 90% of the original activity was retained at 65 and 70 °C for 3 h and 2 h, respectively [107]. This was found to be highly stable in comparison to lipase from *Geobacillus stearothermophilus* with 87.5% original activity retained after 15 min of exposure at 70 °C [138]. Shariff *et al*. [139] reported stability of L2 lipase at 60 °C with a half-life of 2 h. At the same temperature, the enzyme retained more than 40% of its original activity for 3 h and 45 min. The thermostability of L2 lipase was found to be higher than that of *Bacillus stearothermophilus* L1, which was stable at 60 °C for 30 min [40].

9.2. Effect of pH on activity and stability

Bacterial lipases that are active and stable in the wide alkaline pH range (8.0-12.0) represent attractive biocatalysts in many industrial applications. For instance, alkaline lipases that show optimal activities in the pH range from 9.0 to 11.0 have been reported from *Acinetobacter radioresistens*, *Bacillus* sp., *Chromobacterium viscosum*, *Micrococcus* sp., and different species of the genus *Pseudomonas* [141]. In addition, strains of *Bacillus cepacia* ATCC 25609 [142], *Acinetobacter johnsonii* LP28 [24], *Staphylococcus* sp. ESW [218), *Bacillus* sp. LBN 2 [53], and *Bacillus flexus* XJU-1 [143] exhibit alkaline

lipolytic activity. Remarkably, lipases from *Bacillus stearothermophilus* SB-1, *Bacillus atrophaeus* SB-2 and *Bacillus licheniformis* SB-3 are active over a broad pH range (3.0-12.0) [144].

The alkaline lipases exhibit various good stability ranges. This is notable of alkaline lipase from *Acinetobacter calcoaceticus* 1-7 found to be stable in the pH range of 4.0-10.0 and retained 100% of its activity at pH 9.0 for 24 h at 40 °C [25]. In addition, lipase from *Pseudomonas aeruginosa* KM110 was found to be stable in the pH range of 7.0-10.0, retaining 65% activity at pH 8.0 for 1 h at 30 °C [29]. Ekinci *et al*. [145] reported lipase from *Geobacillus stearothermophilus* AH22 that retained more than 70% of its original activity at pH 7.0-10.0 for 30 d at +4 °C. More than 94% activity of lipase from *Anoxybacillus flavithermus* HBB 134 was maintained between pH 6.0 to 11.0 at 25 °C for 24 h [146].

9.3. Kinetics properties of alkaline lipases

In many cases, lipases appear to conform to Michaelis-Menten kinetics, characterized by two parameters, Michaelis-Menten constant (K_m) and maximum velocity (V_{max}). The K_m values of the enzyme vary widely, however for most industrially relevant enzymes, K_m ranges between 10^{-1} and 10^{-5} M in the presence of biotechnologically important substrates [147]. K_m and V_{max} values of 0.16 mM and 0.52 U/mg for *p*-nitrophenyl acetate, 0.02 mM and 1.03 U/mg for *p*-nitrophenyl butyrate, 0.19 mM and 0.72 U/mg for *p*-nitrophenyl octanoate, and 0.55 mM and 0.15 U/mg for *p*-nitrophenyl laurate have been reported for lipase from *Geobacillus stearothermophilus* AH22 [145]. Similarly, Gururaj et al. [78] determined the kinetic properties of *Acinetobacter* sp. AU07 lipase in the presence of different fatty acid esters as substrates. The K_m and V_{max} values were 0.51 mM and 16.98 U/mg for *p*-nitrophenyl butyrate, 0.15 mM and 15.44 U/mg for *p*- nitrophenyl laurate, 0.17 mM and 14.61 U/mg for *p*-nitrophenyl myristate. In addition, K_m and V_{max} values of 0.8 mM and 3.833 mmol/ml/min, respectively were recorded from *Acinetobacter haemolyticus* NS02-30 lipase when using *p*-nitrophenyl palmitate as substrate [60]. Ghori et al. [148] recorded K_m and V_{max} values of 0.345 mM and 7.61 µM/ml/min, respectively in the presence of *p*-nitrophenyl butyrate as substrate from lipase secreted by *Bacillus* sp. FH5.

10. Potential biotechnological applications of alkaline lipases

Microbial lipases constitute an important class of biotechnologically valuable enzymes, mainly due to the versatility in their enzymatic properties and substrate specificity. This versatility makes lipases the enzyme of choice for various potential applications in food, detergent, leather, pharmaceutical, textile, cosmetic, paper industries etc. [149, 2, 20]. Some of the biotechnological applications of alkaline lipases are summarized in Table 4 and discussed in details below:

Table 4: Some potential biotechnological applications of alkaline lipases

Industry	Role	Product or application
Detergent	Removal of fat stains and oil or fatty deposits on clothes	Clean fabrics
Pulp and paper	Removal of pitch from pulp produced during paper making processes	Paper with improved quality
Pollution control	Hydrolysis and trans-esterification of oils and greases	Reduce organic pollutant level
Crude oil industry	Trans-esterification	Biodiesel
Leather	Removal of fats and greases from skins and hides	Cleaner finished products
Dairy foods	Hydrolysis of milk fat; cheese ripening; modification of butter, fat and cream	Flavoring agent in milk, cheese and butter
Beverages	Improved aroma	Alcoholic beverages, e.g. sake wine
Fats and oil industry	Hydrolysis, esterification and inter-esterification	Cocoa butter, margarine, fatty acids, glycerol, mono- and diglycerides
Bakery foods	Enhance flavor content; prolong shelf-life; improve texture and softness	Bread, rolls, pies, muffins, cookies, pastries
Meat and fish	Flavor development; fat removal	Meat and fish products
Food dressings	Quality improvement	Mayonnaise, dressing and whippings
Cosmetics	Esterification	Emulsifiers, moisturizers
Agrochemicals	Esterification, hydrolysis	Herbicides such as phenoxypropionate; chiral building blocks
Pharmaceuticals	Trans-esterification, Hydrolysis	Specialty lipids, digestive aids; intermediates used in the manufacture of medicines

10.1. Lipases in detergent industry

The most commercially significant application of hydrolytic lipases is their addition in detergent, used mainly in household and industrial laundry [150]. Lipases are employed in detergent formulations for the removal of fat stains and oil or fatty deposits on clothes, thus reducing the content of undesirable chemicals. In addition, they are biodegradable, leave no harmful residue, and render no risk to aquatic life [2, 141]. To be a suitable additive in detergents, lipase should exhibit broad substrate specificity, resistant to harsh washing conditions of alkaline pH and temperatures, as well as demonstrate catalytic activity in the presence of various components of detergent formulations [5]. Lipolase from *Thermomyces lanuginosus* represents the first industrial lipase to be introduced into detergent and was commercialized in 1988 by Novo Nordisk. Other lipases including Lumafast and Lipomax from *Pseudomonas mendocina* and *Pseudomonas alcaligenes*, respectively were commercialized by Genencor (now Du Pont) in the 1990s.

Recently, lipases from several microorganisms have been characterized as potent detergent additives [151, 152, 25, 153].

10.2. Lipases in food industry

Fats and oils are indispensable constituents of foods. The nutritional and sensory values as well as physical properties of a triglyceride are greatly influenced by position of fatty acid in the glycerol backbone, the chain length of the fatty acid and its degree of unsaturation etc. [154]. The modification of structure and composition of fat and oil is of great significance in food processing industries that require new economics and green technologies. Microbial lipases that are regiospecific and fatty acid specific are of enormous importance to produce several products in food industries. For instance, lipase-catalyzed reactions can be used to modify and upgrade cheap oil into nutritionally important structured triacylglycerols like cocoa butter substitutes, low calories triacylglycerols and oleic acid enriched oils [155]. Lipases have also been used in foods to modify flavor by synthesis of esters of short chain fatty acids and alcohol, which are known flavor and fragrance compounds [156]. In addition, lipases are used in the removal of fat from meat and fish products to produce lean meat. The fat is removed during the processing of the fish meat by adding lipases, a phenomenon known as bio-lipolysis [157]. Lipases also play a substantial role in the fermentative process of sausage production and to determine changes in long-chain fatty acid released during ripening [2]. Over decades, microbial lipases have been used for refining rice flavor, modifying soybean milk, improving aroma, and enhancing fermentation in apple wine [158].

10.3. Lipases in cosmetics

Immobilized lipase from *Rhizomucor miehei* has been applied as a biocatalyst for the production of cosmetic products including isopropyl palmitate and 2-ethylhexyl palmitate, used as emollient in personal care products such as skin and sun-tan creams, bath oils etc. The use of the enzyme instead of commonly used chemicals gives products of much higher quality with minimum downstream refining. In addition, wax esters (esters of fatty acids and fatty alcohols) produced from catalytic reaction of lipase from *Candida cylindracea* have similar applications in personal care products. Enzymatic production of water-soluble retinol derivatives from immobilized lipase has been reported [159]. Lipases are also used in hair waving preparation and as components of topical anti-obese creams or as oral administration [160, 161, 162].

10.4. Lipases in pulp and paper industry

The pulp and paper industry generates large amounts of lignocellulosic biomass every year. The technology for pulp manufacturing is highly diverse, thus providing numerous opportunities for the application of microbial enzymes. Pitch (hydrophobic components in wood) creates severe problems in paper mill, by

causing sticky deposits in the paper machines as well as holes and spots in the final paper [163]. The enzymatic pitch control technique using lipase has been a routine practice for large-scale paper making process [164]. Lipases are used for the removal of pitch from the pulp produced during paper making processes [165]. Lipases hydrolyze approximately 90% of triglycerides in the pitch into monoglycerides, glycerol and fatty acids, which are less sticky, and highly hydrophilic [163]. In General, lipases in paper industry increase the pulping rate of pulp, increase whiteness and intensity, decrease chemical usage, prolong equipment life, reduce pollution level of wastewater, save energy and time, and reduce composite site [2]. The addition of lipase from *Pseudomonas* sp. KWI-56 to a de-inking composition for ethylene oxide-propylene oxide adduct stearate enhanced whiteness of paper and reduced residual ink spots [166].

10.5. Lipases in bioremediation of lipid-rich wastewater

Lipids are noxious components of industrial and municipal wastewaters, as they contribute greatly to the organic matter of the wastewater and promote the growth of filamentous microorganisms. Therefore, their transformation to innocuous products is imperative. The use of biocatalysts serves as a promising technology for the treatment of high fat-containing wastewater. An alternative to conventional approaches that is attracting growing interest is the use of enzymes, which significantly reduce the level of organic pollutants in the wastewater by means of enzymatic catalysis and enhance better performance of microbial community at the later stage of biological treatment process [167, 168, 169, 170]. Application of lipases from different sources in the treatment of wastewater from lipid-processing factories, dairies, restaurants etc. offers a novel approach in enzyme biotechnology. Thus, making the wastewater amenable to conventional biological treatment [21]. The utilization of a solid enzymatic preparation from *Penicillium restrictum* for the treatment of dairy wastewater with high levels of oil and grease (O & G) has been reported [171]. Results obtained showed 13% higher chemical oxygen demand (COD) removal efficiency with 40% lower accumulation of O & G in flocs; 1.7 times higher biomass concentration, and 1.3 times higher specific oxygen uptake rate.

Immobilization of lipase in insoluble supports is an important tool for the treatment of lipid-rich wastewater. Immobilized lipase has been used for the hydrolysis of O & G contents in pet food industry wastewater, resulting in COD and O & G reduction of 49 and 45%, respectively without pretreatment, and 65 and 64%, respectively, with immobilized lipase treatment [172]. Dumore and Mukhopadhyay [173] used triacylglycerin lipase immobilized in chitosan for the treatment of synthetic oily wastewater. Results recorded revealed approximately 48% and 47% removal efficiencies of O & G and COD, respectively. In another study, Kanmani et al. [174] applied partially purified lipase (*Staphylococcus pasteuri* COM-4A) immobilized on celite carrier for the treatment of coconut oil mill effluent. Result showed 46% and 24%

increase in volatile fatty acids and long-chain fatty acids, respectively with a concomitant reduction in O & G (52%) and COD (32%).

Several lipase-producing microorganisms have been employed individually or as a consortium in the remediation of lipid-containing wastewater. These include *Bacillus stearothermophilus* [175], *Bacillus* sp. [176], *Burkholderia* sp. [177], *Raoultella planticola* [178], *Geotrichum candidum* [179], *Penicillium chrysogenum* [180], *Yarrowia lipolytica* [181]. Consortium of microorganisms including *Pseudomonas aeruginosa*, *Bacillus* sp., and *Acinetobacter calcoaceticus* [182], and *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [183] have been formulated as potential inoculum for the treatment of high strength lipid-rich wastewater.

11. Concluding remarks

Lipases are ubiquitous in nature, often found in plants, animals, and microorganisms. However, microbial lipases are versatile and widely employed for diverse biotechnological applications in food, cosmetics, pulp and paper, detergent industries as well as in environmental bioremediation process. In lieu of the emergent worldwide market and potential applications of microbial lipases, the complex downstream processing steps result in low enzyme yield. Optimization of fermentation parameters through statistical experimental designs is crucial in order to maintain a balance between various components for enhanced lipase production. Immobilization of lipases in appropriate support materials allow continuous use of the enzyme and reduction in the overall costs. The catalytic properties of alkaline lipases provide information on possible identification of enzyme with high activity and stability for exploitation in various industrial and environmental applications. Alternative approaches through metagenomics, site-directed mutagenesis, and cloning and expression of lipase encoding gene in heterologous host should be developed for a robust catalytic activity. In addition, the use of agro-industrial residues and industrial effluents as substrates for lipase production will help in alleviating the costs of the enzyme and serve as pollution control strategies.

Conflicts of interest

The authors declare that there are no conflicts of interest concerning the publication of this paper

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CHAPTER FIVE

INDIGENOUS *ACINETOBACTER* SP. ISOLATED FROM LIPID-RICH WASTEWATER IN DURBAN PRODUCED THERMOSTABLE GLYCOPROTEIN BIOEMULSIFIERS

This chapter has been submitted in the current format to the '3 Biotech'

Indigenous *Acinetobacter* sp. isolated from lipid-rich wastewater in Durban produced thermostable glycoprotein bioemulsifiers

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Abstract The potential of indigenous bacteria isolated from lipid-rich wastewater to produce bioemulsifier was investigated using 2% (v/v) olive oil as carbon source. Of the eighteen bioemulsifier/biosurfactant-producing bacterial isolates, two demonstrated no significant reduction in surface tension, and were classified as bioemulsifier producers. The isolates were identified as *Acinetobacter* sp. AB9-ES and *Acinetobacter* sp. AB33-ES based on PCR amplification and analysis of their 16S rRNA gene sequences. Bioemulsifier production by these organisms was found to be growth-associated, with maximum emulsifying activities of 83.8 ± 0.17% (at 168 h) and 80.8 ± 0.29% (at 120 h) observed for strains AB9-ES and AB33-ES, respectively. Bioemulsifier yields of 4.52 ± 0.03 and 4.31 ± 0.04 g/L was obtained for strains AB9-ES and AB33-ES, respectively. FT-IR analysis revealed the glycoprotein nature of both bioemulsifiers. The bioemulsifier from strain AB9-ES (XB9) and strain AB33-ES (YB33) formed stable emulsions only with edible oils with highest emulsification indices of 79.6 and 67.9%, respectively obtained against sunflower oil. The emulsifying activity of the bioemulsifiers was stable over broad range of temperature (4-121 ºC), moderate salinity (1-6%) and pH (5.0-10.0), suggesting the potential biotechnological applications of these bioemulsifiers, especially in the remediation of oil-polluted sites.

Keywords *Acinetobacter* sp.; Bioemulsifier; Emulsifying activity; Lipid-rich wastewater; Remediation

Introduction

Lipid-rich wastewater is characterized by the presence of fats, oils and greases (FOGs) as well as vast array of dissolved organic and/or inorganic substances in suspension at high concentrations (Adulkar and Rathod 2015). The discharge of untreated and inadequately treated lipid-rich wastewater increases every year due to rapid urbanization and industrial growth. This poses serious threat to both terrestrial and aquatic ecosystems (Rupani et al. 2010). Among the most efficient treatment approaches is the addition of microbial

surface active compounds (SACs) to the wastewater. This disperses the lipids and other pollutants in the wastewater, and accelerate their mineralization through increased bioavailability and subsequent uptake by direct cell contact (Makkar et al. 2011; Franzetti et al. 2012).

Surface active compounds are amphiphilic molecules which form micelles and partition at the interface between fluid phases with varied degrees of polarity (Santos et al. 2016). They are classified into two groups: low molecular and high molecular weight SACs. The low molecular weight compounds, also known as biosurfactants form micelles at the interface of immiscible liquids by reducing surface and interfacial tension, and blocking hydrogen bonding and hydrophilic/hydrophobic interactions (Luna et al. 2012). They consist of glycolipids, fatty acids, phospholipids, neutral lipids amongst others with molecular mass ranging between 500-1500 Da (Banat et al. 2010). Conversely, bioemulsifiers are high molecular weight compounds that emulsify two immiscible liquids even at low concentrations, without necessarily reducing surface or interfacial tension (Uzoigwe et al. 2015). They consist of polysaccharides, lipopolysaccharides, proteins, glycoprotein or lipoproteins, which confer upon them better emulsifying potential and ability to stabilize emulsions (Rosenberg and Ron 1999; Uzoigwe et al. 2015).

Bioemulsifiers are produced by a wide variety of microorganisms including bacteria, yeast, and fungi using different substrates such as carbohydrates, hydrocarbons, vegetable oils and glycerols for cell growth (Kitamoto et al. 2002; Sarubbo et al. 2007). However, bioemulsifier production has been reported to be a common phenomenon among members of the genus *Acinetobacter* (Rosenberg 1986). Examples include emulsan, an extracellular polyanionic bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1 (Rosenberg and Ron 1999). Others include alasan from *Acinetobacter radioresistens* (Navon-Venezia et al. 1995), biodispersan from *Acinetobacter calcoaceticus* A2 (Rosenberg et al. 1988). Many strains of *Acinetobacter* isolated from contaminated soil, mud, marine water, fresh water and human skin have been reported to be bioemulsifier producers (Sar and Rosenberg 1983; Foght et al. 1989; Patil and Chopade 2001; Phetrong et al. 2008). However, few studies have been carried on bioemulsifier-producing *Acinetobacter* sp. from lipid-rich wastewater (Saisa-ard et al. 2013).

In contrast to synthetic surfactants, bioemulsifiers are environmentally-friendly, biocompatible with less toxicity and higher biodegradability, and specifically active at extreme temperature, pH and salinity. In addition, bioemulsifier can be produced from renewable cheaper substrates through fermentation processes (Pansiripat et al. 2010). Due to diverse functional properties such as emulsification, wetting, foaming, cleansing, phase separation, surface activity and reduction in hydrocarbon viscosity, bioemulsifiers are best employed in bioremediation, enhanced oil recovery, clean-up of oil-contaminated pipes and vessels, among others (Martinez-Checa et al. 2007; Dastgheib et al. 2008).

In spite of their immense applications, large scale production of bioemulsifier has been limited due to low yield and high production costs (Banat et al. 2014). Alternative approaches for ameliorating the aforementioned challenges include the selection of efficient strains for optimum bioemulsifier production (Marchant et al. 2014). Exploration of native strains could be of great significance for bioemulsifier production, since native strains can be assumed to perform better in their native environment than exotic strains. To the best of our knowledge, this is the first report on bioemulsifier production from *Acinetobacter* sp. isolated from lipid-rich wastewater in South Africa. This study therefore characterized glycoprotein bioemulsifier produced by *Acinetobacter* sp. isolated from lipid-rich wastewater, in Durban, South Africa against different hydrophobic substrates. The stability of the emulsifying activity of the bioemulsifiers at extreme temperature, pH and salinity was also determined in order to ascertain their possible industrial and environment applications.

Materials and methods

Sample collection and bacterial isolation procedures

Lipid-rich wastewater samples were collected from four different sites namely; an industrial wastewater treatment plant (WWTP), slaughterhouse (SH), edible oil mill (EOM) and soap (SP) industries, all in the KwaZulu-Natal (KZN) province, South Africa into sterile 500 ml Schott bottles, and transported immediately using an ice-packed box to the laboratory for further analysis. Samples of lipid-rich wastewater (10 ml) were transferred to 250 ml Erlenmeyer flasks containing 100 ml mineral salt medium (MSM) consisting (in g/L): 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.2 $MgSO_4.7H_2O$, 0.02 $CaCl_2.2H_2O$ and 0.05 $FeCl_3.6H_2O$ supplemented with 2% olive oil, and then incubated at 37 ºC for 48 h at 120 rpm. Cultures (5 ml) were subsequently transferred into the second flask and cultivated under the same conditions. This process was repeated four times. Samples (0.1 ml) of the last enrichment cultures were serially diluted, plated onto nutrient agar (NA) and incubated at 37 ºC for 24 h. Pure cultures obtained by sub-culturing on fresh NA plates were preserved at -80 ºC in a storage medium (500 µl of 80% sterile glycerol and 500 µl culture suspension).

Screening of bacteria for bioemulsifier/biosurfactant production

Mineral salt medium supplemented with 2% (w/v or v/v) glucose, diesel, olive oil, sunflower oil, canola oil, castor oil or rice bran oil as sole carbon and energy sources was used. The pH of the medium was adjusted to 7.0 using 1 N NaOH or 1 N HCl. Un-inoculated control flask was prepared for each carbon source. Homogenous bacterial culture [1% (v/v) of OD_{600nm} = 1] was inoculated into the MSM in 250 ml Erlenmeyer flasks and cultures grown at 37 ºC and 120 rpm for 7 d. Bacterial growth was evaluated at 600

nm using UV-Vis spectrophotometer (UVmini-1240, Schimadzu, Australia). Cultures with higher increase in OD_{600nm} when compared to that of control flask were scored positive (+) while those exhibiting no difference in turbidity were taken as no growth (-). Culture broth was centrifuged at 8000 rpm for 20 min at 25 ºC. The obtained culture supernatants from respective test carbon sources were evaluated for bioemulsifier/biosurfactant production using oil spreading test (OST) (Morikawa et al. 2000), emulsification assay (Cooper and Goldenberg 1987), and surface tension (ST) measurement (Gudiña et al. 2012). Effect of the carbon sources was studied by comparing bioemulsifier/biosurfactant production after growth on the different carbon sources. All experiments were done in triplicate.

Amplification and sequencing of 16S rRNA gene and phylogenetic analysis

Genomic DNA was isolated from bacterial cultures using boiling method described by (Akinbowale et al. 2007) with some modifications. Amplification of the 16S rRNA gene fragment of the bioemulsifier-producing bacteria was carried out using universal primers F-5'-CAGGCCTAACACATGCAAGTC-3' and R-5'-GGGCGGTGTGTACAAGGC-3' (Marchesi et al. 1998). The PCR reaction mixture consisted of 1× buffer, 1mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 µM of each primer, 2 U of *Taq* DNA polymerase and 2 µl template DNA to a total volume of 25 µl. The mixture was subjected to amplification using a thermocycler (Bio-Rad T100, Singapore) with the following conditions: initial denaturation at 95 ºC for 5 min, followed by 30 cycles of denaturation at 95 ºC for 1 min, annealing at 55 ºC for 1 min, extension at 72 ºC for 1min, 30 sec, and final extension at 72 ºC for 5 min. Amplification products were observed by electrophoresis in a 1.5% (w/v) agarose gel at 100 V for 45 min in a 1% TAE buffer. After staining in 1 mg/ml ethidium bromide for 15 min, products were viewed by UV illumination (Syngene, UK), and then sequenced (inqaba Biotechnical Industries, Pretoria, South Africa). The resulting sequences were compared with sequences in the GenBank database of National Centre for Biotechnology Information (NCBI) using the Nucleotide Basic Local Alignment Search Tool (BLAST N) program (www.ncbi.n1m.nih.gov/BLAST). The sequences were aligned using the multiple sequences alignment tool, Clustal W. Phylogenetic tree was constructed through Molecular Evolutionary Genetics analysis (MEGA) version 6.0 software (Tamura et al. 2013) using a neighbor-joining method.

Kinetics of growth and bioemulsifier production

Acinetobacter sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES were subjected to growth and bioemulsifier production kinetic study in a MSM (100 ml) supplemented with 2% (v/v) of filter-sterilized (0.2 µm) olive oil. The pH of the medium was adjusted to 7.0 using 1 N NaOH or 1 N HCl. Bacterial culture [1% (v/v) of OD_{600nm} = 1] was inoculated into the medium in 250 ml Erlenmeyer flasks, and then incubated at 37 ºC for 168 h at 120 rpm. Samples were taken at 24-h intervals to determine bacterial growth, emulsifying activity

and ST. Un-inoculated production medium was used as a control. Bacterial growth was determined by optical density measurement at 600 nm using UV-Vis spectrophotometer (UVmini-1240, Schimadzu, Australia). Thereafter, samples were centrifuged at 8000 rpm, 25 ºC for 20 min. ST and emulsification index, EI_{24} (%) of the culture supernatants were determined, as described previously. All evaluations were carried out in triplicate.

Extraction and partial purification of bioemulsifier

Bioemulsifier was precipitated from the culture supernatant obtained by centrifugation at 8000 rpm for 20 min with three volumes of cold ethanol followed by incubation overnight at -20 ºC. Thereafter, the precipitated bioemulsifier was re-dissolved in distilled water, dialyzed in cellulose tube membrane (cut-off: 12.4 KDa) (Sigma-Aldrich, USA) for 24 h against distilled water (1%, w/v) and then lyophilized. The obtained product was then weighed (Joshi et al. 2008).

Chemical composition of bioemulsifier

Standards methods were used for determining the chemical composition of bioemulsifier. Carbohydrate content was determined by the phenol-sulfuric acid method of Dubois et al. (1956) using D-glucose as a standard. Protein content was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Lipid content was estimated by using the Folch extraction method (Folch et al. 1957). All experiments were carried out in triplicate.

Fourier transforms infrared spectroscopy (FTIR) analysis

Functional groups of the bioemulsifier samples were elucidated by FT-IR. The FT-IR spectra with a resolution of 4 cm^{-1}were collected in the wavenumber range of 3500-500 $cm^{-1}$with 32 scans using a Bruker ALPHA P platinum FT-IR spectrometer. The analysis of IR spectra was carried out using OPUS 6.5 (Bruker Optics) software. All measurements were recorded at room temperature.

Effect of different hydrophobic substrates on bioemulsifier activity

The emulsifying activity of the bioemulsifier (1%, w/v) was determined in the presence of different hydrophobic substrates including edible oils (sunflower oil, canola oil, castor oil, rice bran oil), aromatic hydrocarbons (benzene, xylene, toluene), aliphatic hydrocarbons (heptane, dodecane, hexane) as well as hydrocarbon mixtures (motor oil, kerosene, diesel). This was done by vortexing 2 ml of bioemulsifier solution with the same amount of respective test hydrocarbons for 2 min. The mixture was then allowed to stand for 24 h. EI_{24} (%) was calculated as the height of the emulsion layer divided by height of the total

mixture multiplied by 100. The results were compared with those obtained with SDS. All evaluations were carried out in triplicate.

Bioemulsifier stability studies

Effect of temperature, pH and salinity on bioemulsifier activity

The influence of temperature, pH and salinity on the emulsifying activity of bioemulsifier was investigated against sunflower oil (substrate that gave highest emulsification index) by pretreating the obtained bioemulsifier solution (1%, w/v) at varying temperatures, pH and NaCl concentrations. Bioemulsifier solution was exposed to different temperatures (4, 30, 40 50, 60, 70, 80, 90, 100, 110 and 121 ºC) for 20 min and then cooled to room temperature. The effect of pH was assessed by adjusting the pH of the bioemulsifier solution to different pH ranges (2-13) using 1 N HCl or 1N NaOH solution. Influence of salinity was investigated by supplementing bioemulsifier solution with different NaCl concentrations (1-10%, w/v). EI_{24} (%) was measured as described previously against sunflower oil. SDS was used as a positive control. All experiments were carried out in triplicate.

Results and discussion

Isolation, screening and identification of bioemulsifier-producing bacteria

A wide variety of industries produces effluent rich in lipids. Effluents discharged by these industries present potential problems in terms of wastewater management due to their high FOG contents (Brooksbank et al. 2007). In order to isolate indigenous bacteria capable of degrading the FOG through the production of extracellular SACs, lipid-rich wastewater samples were collected from different sites including WWTP, SH, EOM and SP industries, all in the KZN province of South Africa. A total of 20 morphologically distinct bacteria including 5 from WWTP samples, 7 from SH samples, 5 from EOM samples, and 3 from SP samples were isolated from the lipid-rich wastewater. These bacterial isolates were screened for the ability to produce SACs by cultivating them in MSM supplemented with filter-sterilized glucose, diesel, olive oil, sunflower oil, canola oil, castor oil or rice bran oil as carbon sources. Bacterial growth was determined with respect to each carbon source. The obtained culture supernatants were tested for bioemulsifier/biosurfactant production using OST and emulsification assay. Eighteen bacterial isolates showing positive results for both tests were designated as bioemulsifier/biosurfactant producers (Table 1). All these bacterial isolates tested positive for bioemulsifier/biosurfactant production in the presence of olive oil as carbon source. However, of the 18 bacterial isolates, 5 tested positive in the presence of glucose, 4 in the presence of sunflower oil, and 2 in the presence of canola oil, castor oil and rice bran oil. Despite the growth of 5 bacterial isolates on diesel, bioemulsifier/biosurfactant production was not observed on this carbon source.

Olive oil (2% v/v) was chosen as carbon source for further assays, since it was mostly utilized for growth and bioemulsifier and biosurfactant production by all the tested bacterial isolates. Haba et al. (2000) reported the production of rhamnolipid from *Pseudomonas* sp. in the presence of olive oil as carbon source. Surface tension was measured on the culture supernatants of the 18 bacterial isolates tested positive for SACs production in the preliminary screening in order to distinguish between biosurfactant and bioemulsifier producers. Ability of an organism to reduce ST below a threshold limit of 40 mN/m is an indication of its surface activity (Cooper 1986).

Table 1 Growth and bioemulsifier/biosurfactant production by bacterial isolates cultivated in MSM supplemented with different carbon sources[a].

Isolate	Source	Glucose		Diesel		Olive oil		Sunflower oil		Canola oil		Castor oil		Rice bran oil	
		G	B	G	B	G	B	G	B	G	B	G	B	G	B
SM5	EOM	+	-	-	-	+	+	+	-	+	-	+	-	+	-
SM7	EOM	+	-	-	-	+	+	+	-	+	-	-	-	+	-
SM19	EOM	+	-	-	-	+	+	+	-	+	-	-	-	+	-
SM20	EOM	+	-	-	-	+	+	+	-	+	-	-	-	+	-
SM23	EOM	+	-	-	-	+	+	+	-	+	-	-	-	+	-
Ab9-ES	SH	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Ab22-ES	SH	+	+	+	-	+	+	+	-	+	-	+	-	+	-
Ab27-ES	SH	+	+	+	-	+	+	+	+	-	-	-	-	-	-
Ab33-ES	SH	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Ab35-ES	SH	+	-	-	-	+	+	+	-	-	-	-	-	-	-
Ab38-ES	SH	+	+	-	-	+	+	+	+	+	-	-	-	+	-
Ab42-ES	SH	+	-	-	-	+	+	+	-	-	-	-	-	-	-
As5	WWTP	+	-	-	-	+	+	+	-	-	-	-	-	-	-
As33	WWTP	+	-	-	-	+	+	+	-	-	-	+	-	-	-
As53	WWTP	+	-	-	-	+	+	-	-	+	-	-	-	-	-
As72	WWTP	+	-	-	-	-	-	+	-	-	-	-	-	-	-
Ow9	WWTP	+	-	+	-	-	-	+	-	-	-	-	-	-	-
SE9	SP	+	-	-	-	+	+	+	-	-	-	-	-	-	-
SE13	SP	+	-	-	-	+	+	-	-	+	-	-	-	-	-
SE26	SP	+	-	-	-	+	+	+	-	+	-	-	-	-	-

[a]2% (w/v, v/v); Growth (G) and bioemulsifier/biosurfactant (B) production: +, positive; -, negative; EOM = edible oil mill industry; SH = slaughterhouse; WWTP = wastewater treatment plant; SP = soap industry

Amongst the tested bacterial isolates, 16 decreased the ST of the culture supernatants below 40 mN/m while 2 (Ab9-ES and Ab33-ES) produced ST above 40 mN/m (48.4 ± 1.9 and 46.4 ± 2.2 mN/m, respectively) (Table 2). This indicated that bacterial isolates Ab9-ES and Ab33-ES are bioemulsifier producers and not biosurfactant producers. Low molecular weight biosurfactant reduced ST below 40 mN/m (Mulligan 2005) while high molecular weight bioemulsifier formed and stabilized emulsions without remarkable ST

reduction (Batista et al. 2006). Therefore, Ab9-ES and Ab33-ES were classified as bioemulsifier producers and selected for further study.

Table 2 Surface tension of bacterial cultures grown in MSM supplemented with 2% (v/v) olive oil as carbon source

Isolate	Source	ST (mN/m)
SM5	EOM	34.7 ± 1.5
SM7	EOM	39.1 ± 1.3
SM19	EOM	33.0 ± 2.1
SM20	EOM	35.6 ± 1.5
SM23	EOM	38.5 ± 1.2
Ab9-ES	SH	48.4 ± 1.9
Ab22-ES	SH	39.5 ± 1.9
Ab27-ES	SH	31.8 ± 2.2
Ab33-ES	SH	46.4 ± 2.2
Ab35-ES	SH	30.9 ± 1.4
Ab38-ES	SH	32.4 ± 2.5
Ab42-ES	SH	31.2 ± 1.5
As5	WWTP	30.9 ±1.3
As33	WWTP	30.9 ± 1.2
As53	WWTP	37.4 ± 1.9
As72	WWTP	ND[b]
Ow9	WWTP	ND[b]
SE9	SP	30.2 ± 1.8
SE13	SP	33.7 ± 1.3
SE26	SP	32.8 ± 0.8

ND[b]: The value was not determined because no growth was observed in the production medium

Identification of strain Ab9-ES and Ab33-ES based on 16S rRNA gene sequence showed that strain Ab9-ES and AB33-ES exhibit close homology (99% and 98%, respectively) to *Acinetobacter soli* KSM2 (KP297393.1). These study strains were therefore designated as *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES. The relatedness of these strains to other bioemulsifier-producing *Acinetobacter* sp. is illustrated in the constructed phylogenetic tree (Fig. 1).

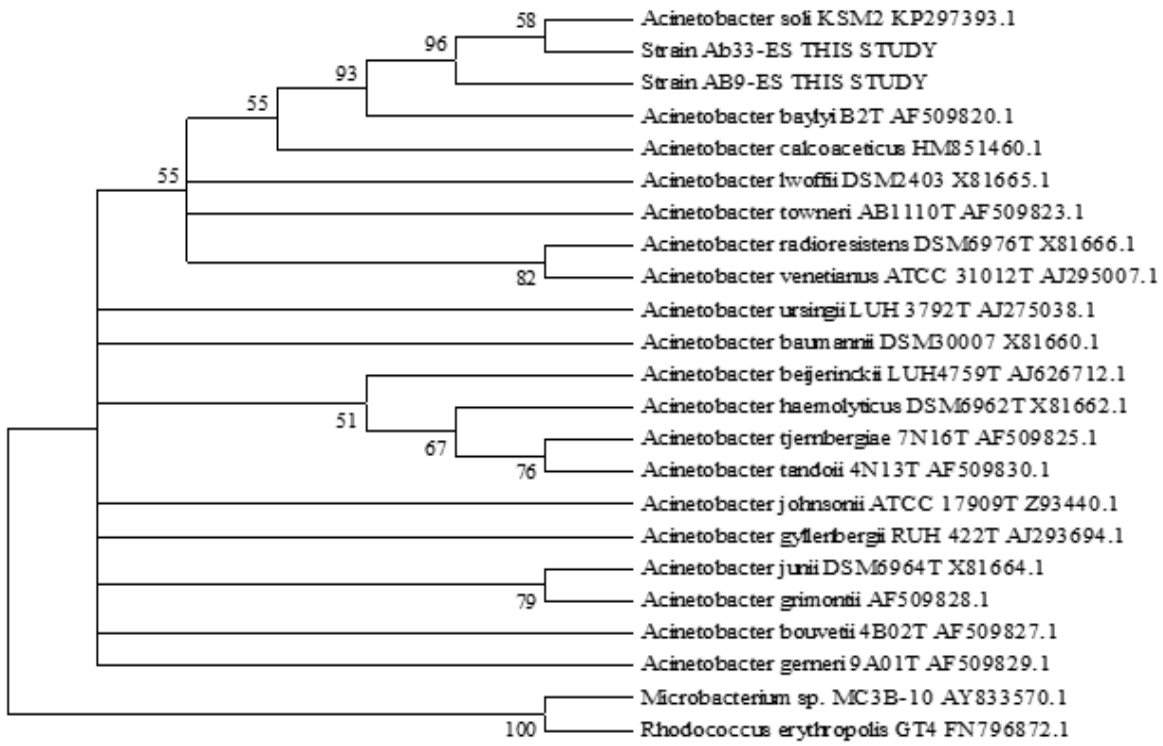

Fig. 1 Phylogenetic neighbor-joining tree based on 16S rRNA gene sequences of *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES showing their relatedness to other bioemulsifier-producing bacterial strains.

Kinetics of growth and bioemulsifier production

Acinetobacter sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES were cultivated in MSM supplemented with 2% (v/v) olive oil as carbon source for the production of bioemulsifier. Growth of these strains was monitored at 24-h intervals over 7 d in order to ascertain exact phase at which optimum bioemulsifier production was achieved. The growth, ST and emulsifying activity profiles during cell cultivation are presented in Fig. 2. In both *Acinetobacter* species, bioemulsifier production was found to be growth- associated, as parallel relationship was observed between cell growth and emulsifying activity. Growth-associated biosurfactant/bioemulsifier production from *Selenomonas ruminantium* CT2 and *Paenibacillus* sp. #510 has been reported (Saimmai et al. 2013; Gudiña et al. 2015). Maximum cell growth (OD_{600nm} = 2.46 ± 0.13) at 144 h and (OD_{600nm} = 2.28 ± 0.1) at 120 h was attained by *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES, respectively.

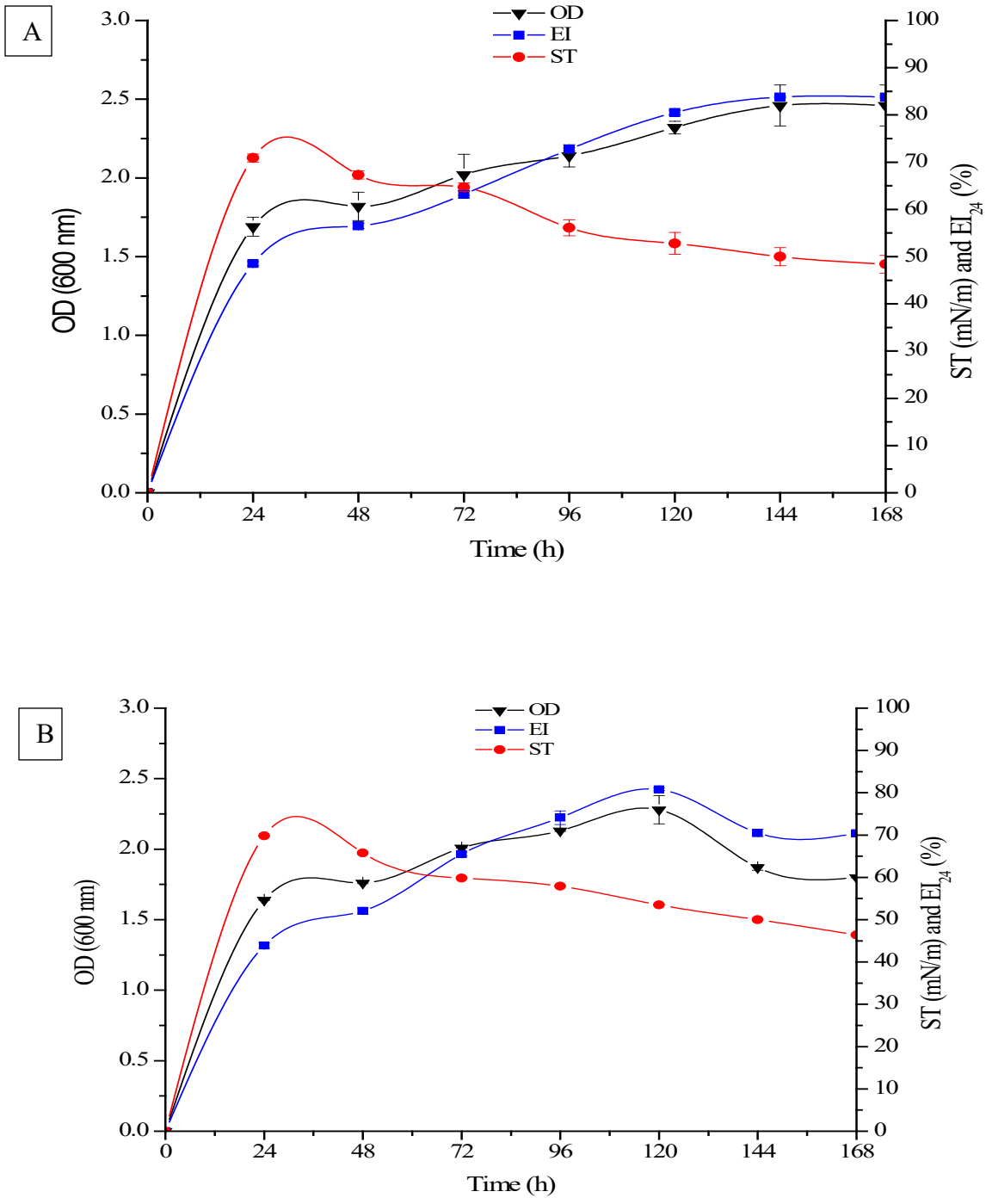

Fig. 2 Kinetics of microbial growth and bioemulsifier production in terms of time course profile of cell growth (OD), ST and emulsifying activity of (A) *Acinetobacter* sp. Ab9-ES and (B) *Acinetobacter* sp. Ab33-ES grown in production medium supplemented with 2% olive oil as carbon source. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Maximum bioemulsifier production was recorded at different phases of growth in both studied *Acinetobacter* sp. In *Acinetobacter* sp. Ab9-ES, the EI_{24} reached the highest value of 83.8 ± 0.17% at 168 h during stationary phase of growth. This is in agreement with the findings of Pornsunthorntawee et al. (2008) where maximum biosurfactant production by *B. subtilis* PT2 was observed during stationary phase. However, *Acinetobacter* sp. Ab33-ES attained highest emulsifying activity of 80.8 ± 0.29% at 120 h during late exponential phase, after which the emulsifying activity decreased probably due to the degradation of the bioemulsifier. Maximum emulsifying activity of 60 ± 1% of *Aeribacillus pallidus* YM-1 has been reported during exponential phase (Zheng et al. 2012). A slight reduction in ST from 70.9 ± 0.85 to 48.4 ±1.9 mN/m and 70.0 ± 2.5 to 46.4 ± 2.2 mN/m was observed in the culture supernatants of *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES, respectively as the incubation period increased from 24 to 168 h (Fig. 2). Ethanol precipitation of culture supernatants gave rise to the formation of dried whitish bioemulsifier powders, denoted as bioemulsifier XB9 and YB33 with a resultant production yields of 4.52 ± 0.03 and 4.31 ± 0.04 g/L by *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES, respectively.

Composition of bioemulsifier

Preliminary studies on chemical composition of the bioemulsifier XB9 and YB33 were conducted. Results showed that XB9 consisted predominantly of carbohydrates (65.5%) and proteins (31%) while YB33 composed of carbohydrates (67.8%) and proteins (25%). This was further confirmed by FT-IR analysis (Fig. 3). The spectra obtained in both bioemulsifiers exhibit some similarities, especially broad absorption bands at 3275.86 cm^{-1} (XB9) and 3287.13 cm^{-1}(YB33) which revealed the presence of hydroxyl and amine groups typical of carbohydrates and peptides, respectively. Absorption peaks in higher frequencies have been reported to be peculiar features of polysaccharides (Stuart 2004). The presence of weak C-H stretching bands at 2926.78 and 2978.14 cm^{-1} in YB33 characterized symmetric stretch (γC-H) of CH_2 and CH_3 groups of aliphatic chains, and O-H content of polysaccharides. In addition, the distinct absorption band at 1646.44 cm^{-1} in YB33 indicates the stretching mode of CO-N bond of acetamido groups of N-acetylated sugars found mostly in microbial polysaccharides (Christensen 1989), as well as N-H bend of primary amines. Similarly, a band at 1634 cm^{-1}in XB9 also correspond to N-H bend. Weak absorption bands at 1459.62 and 1405.40 cm^{-1} in XB9 correspond to C-H stretch of aliphatic amines. Furthermore, absorption bands at 1059.54, 978.69, 861.74 and 724.11 cm^{-1} indicate C-N, C-H, N-H and C-H groups, respectively of aliphatic amines. Similarly, in the case of YB33, absorption bands stretching between 877.52-1453.07 cm^{-1} are typical of C-H and C-N functional groups of amino sugars within the polysaccharides. The FT-IR spectra obtained in the present study suggest that the bioemulsifier XB9 and YB33 composed predominantly of polysaccharides and proteins.

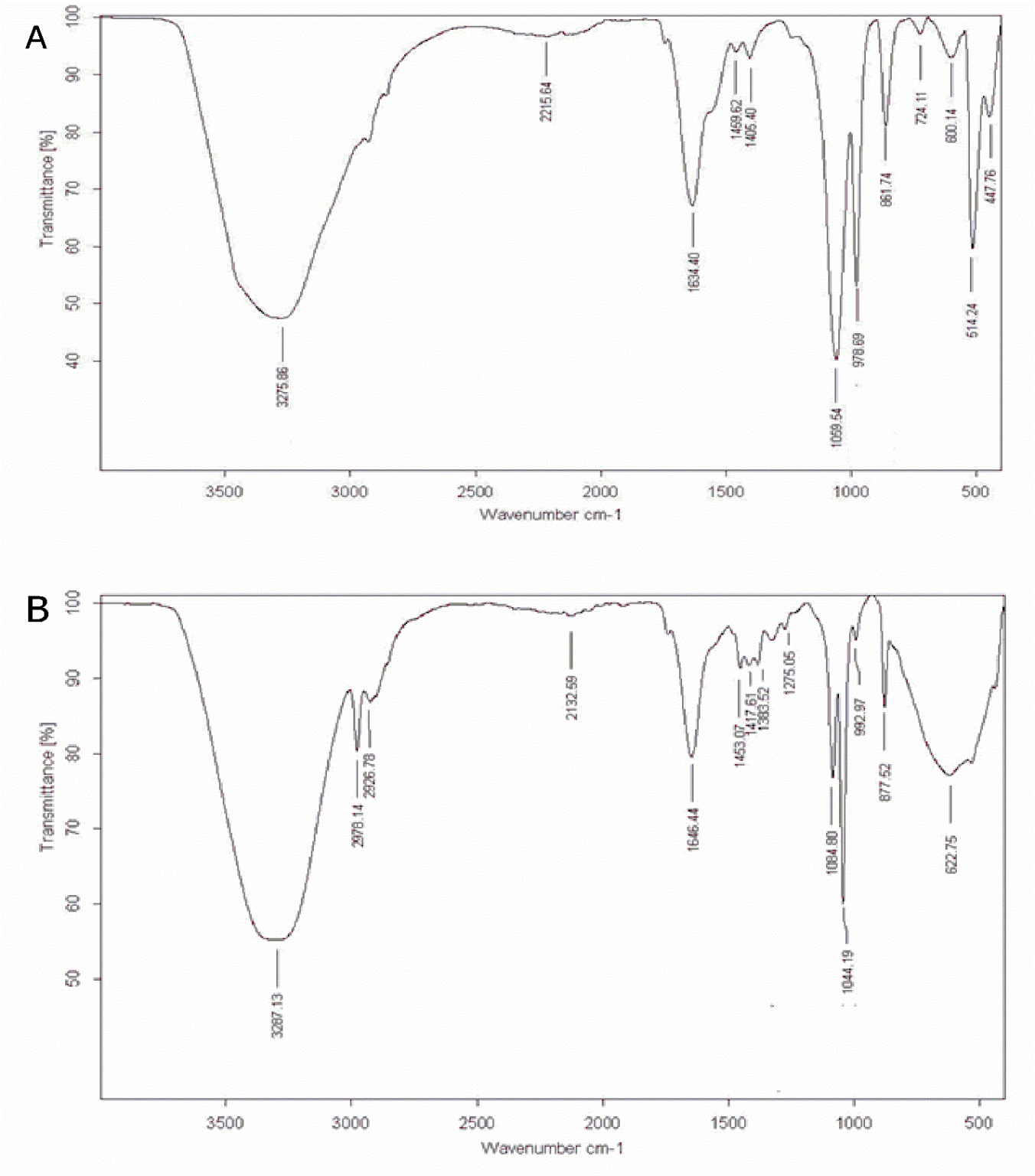

Fig. 3 FT-IR spectra of bioemulsifier (A) XB9 and (B) YB33 produced by *Acinetobacter* sp. Ab9-ES and *Acinetobacter* sp. Ab33-ES, respectively.

Based on the above results, both bioemulsifiers were tentatively identified as glycoproteins. Similar infrared spectra have been reported for bioemulsifier produced by *Ochrobactrum pseudintermedium* strain C1 (Bhattacharya et al. 2014) and *Solibacillus silvestris* AM1 (Markande et al. 2013).

Effect of hydrophobic substrates on emulsifying activity

The ability of the bioemulsifiers to form stable emulsions against wide range of hydrophobic substrates including edible oils (sunflower oil, canola oil, castor oil, rice bran oil), aromatic hydrocarbons (benzene, xylene, toluene), aliphatic hydrocarbons (heptane, dodecane, hexane) and mixed hydrocarbons (motor oil, kerosene, diesel) was investigated, and results are presented in Fig. 4. The EI_{24} values were compared to that of a known surfactant, SDS. The bioemulsifier XB9 and YB33 exhibited higher emulsification activity against sunflower oil, canola oil, castor oil and rice bran oil while less stable emulsions were formed with other hydrocarbons, especially kerosene (13.7%) and diesel (6.9%). This implied that, the bioemulsifiers were able to emulsify edible oils efficiently. Amongst edible oils, higher EI_{24} values of 79.6 and 67.9% were obtained for bioemulsifier XB9 and YB33, respectively towards sunflower oil. Biosurfactant produced by a novel *Pseudomonas* sp. 2B was reported to demonstrate maximum emulsifying activity of 84% towards sunflower oil (Aparna et al. 2012). Emulsion-stabilizing capacity of an emulsifier is the ability to maintain at least 50% of the original volume of the emulsion for 24 h (Freitas et al. 2009). The emulsifying activity of the bioemulsifier XB9 and YB33 was substrate-dependent. Most microbial surface active compounds are substrate-specific and possess ability to emulsify different hydrocarbons at varying rates (Luna-Velasco et al. 2007).

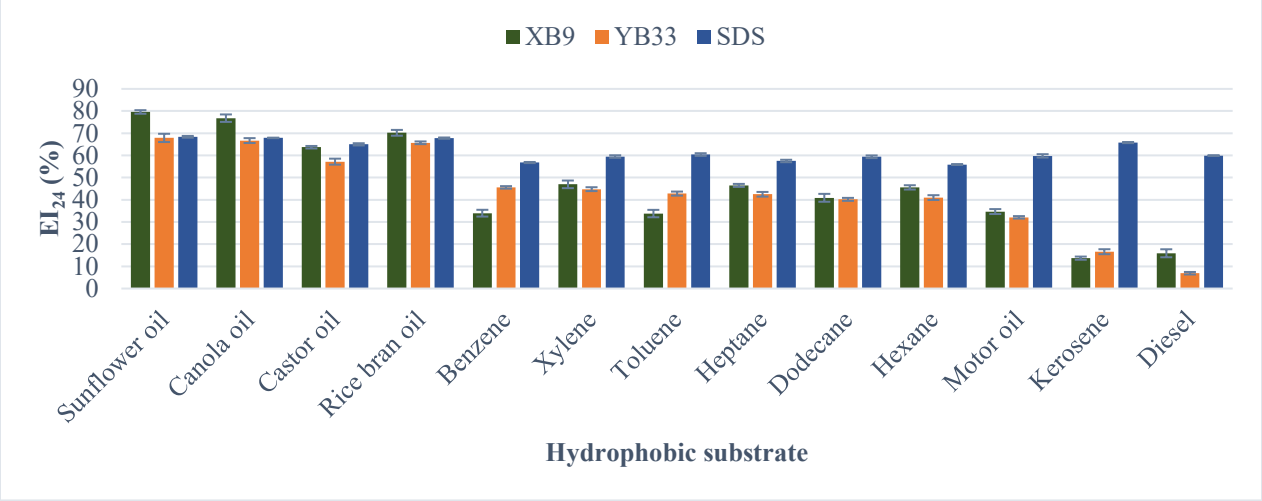

Fig. 4 Emulsifying activity of bioemulsifier XB9 and YB33 on various hydrophobic substrates in comparison to SDS. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Other researchers have reported similar results on hydrocarbon substrate specificity of bioemulsifiers (Sarubbo et al. 2001). The results show that bioemulsifier XB9 and YB33 tested in this study have high ability to stabilize emulsions with edible oils, suggesting their potentials as cleaning and emulsifying agents in the food industry as well as in the treatment of lipid-rich wastewater.

Effect of temperature, pH and salinity on emulsifying activity

Effectiveness of bioemulsifier is dependent on their emulsifying activities over a broad range of temperature, pH and salinity (Markande et al. 2013). The effect of various temperatures, pH and NaCl concentrations on emulsifying activity of the bioemulsifiers is shown in Fig. 5. Both bioemulsifiers (XB9 and YB33) maintained stable emulsions over broad range of temperatures (4-121 ºC), except at 121 ºC, at which YB33 had a lower EI_{24} value (43.6%). The emulsifying activity increases from 4 to 50 ºC and 4 to 70 ºC for XB9 and YB33, respectively (Fig. 5A). Slight decrease in EI_{24} values were observed beyond these temperatures. This may be due to denaturation of protein component of the bioemulsifier during heat treatment. Highest emulsifying activity of 77.3 ± 1.45% was observed at 50 ºC for XB9 whereas YB33 attained optimum activity of 73.6 ± 0.46% at 70 ºC. Optimum temperature of 50 ºC has been reported for high emulsifying activity of microbactan produced by *Microbacterium* sp. MC3B-10 (Camacho-Chab et al. 2013). Heat stability of liposan produced by *Candida lipolytica* has also been reported at temperature up to 70 ºC (Cirigliano and Carman 1984). It is remarkable that bioemulsifier XB9 retained fairly good emulsifying activity (52.6%) even at 121 ºC. Such stability at extreme temperature has been reported for a lipopeptide biosurfactant produced by *Brevibacterium aureum* MSA 13 (Kiran et al. 2010). Hence, it could be concluded that bioemulsifiers are thermostable in nature.

The stability of the bioemulsifier XB9 and YB33 was investigated over broad pH values (2.0 to 13.0), and emulsifying activity was measured (Fig. 5B). The bioemulsifier XB9 and YB33 demonstrated emulsion-stabilizing capacities over pH ranges of 5.0-10.0 and 5.0-9.0, respectively. Highest emulsifying activities of 74.5% and 72% were attained by XB9 and YB33, respectively at pH 7.0, and these were found to be higher than that exhibited by synthetic surfactant, SDS. Pronounced reduction in activity was recorded at pH values higher than 7.0. This may probably be due to some structural alterations of the bioemulsifiers under extreme pH conditions. Bioemulsifier from *Bacillus subtilis* has been reported to be active at pH 7.0 with a significant reduction in activity at pH value above 7.0 (Cooper and Goldenberg 1987). Optimum emulsifying activity (EI_{24} 84%) by biosurfactant produced by a novel *Pseudomonas* sp. 2B was observed at pH 7.0 (Aparna et al. 2012) The synthetic surfactant, SDS maintained stable emulsion over a wide-spectrum of temperatures and pH studied with little differences in EI_{24} values observed.

Investigation of the effect of salinity on emulsifying activity of the bioemulsifiers XB9 and YB33 at NaCl concentrations ranging from 1-10% (w/v) indicated the stability of both bioemulsifiers up to 6% salinity (Fig. 5C). Similar results have been observed previously with bioemulsifier produced from *Ochrobactrum pseudintermedium* strain C1 (Bhattacharya et al. 2014). Maximum activity (57.4%) was observed at NaCl concentration of 3% for XB9. On the contrary, YB33 demonstrated highest activity of 61.2% at NaCl concentration of 5%. Beyond these salinity levels, the emulsifying activity declined drastically. Stability of these bioemulsifiers at NaCl concentrations up to 6% warrant intensified bioprospection in the remediation of polluted intertidal zone.

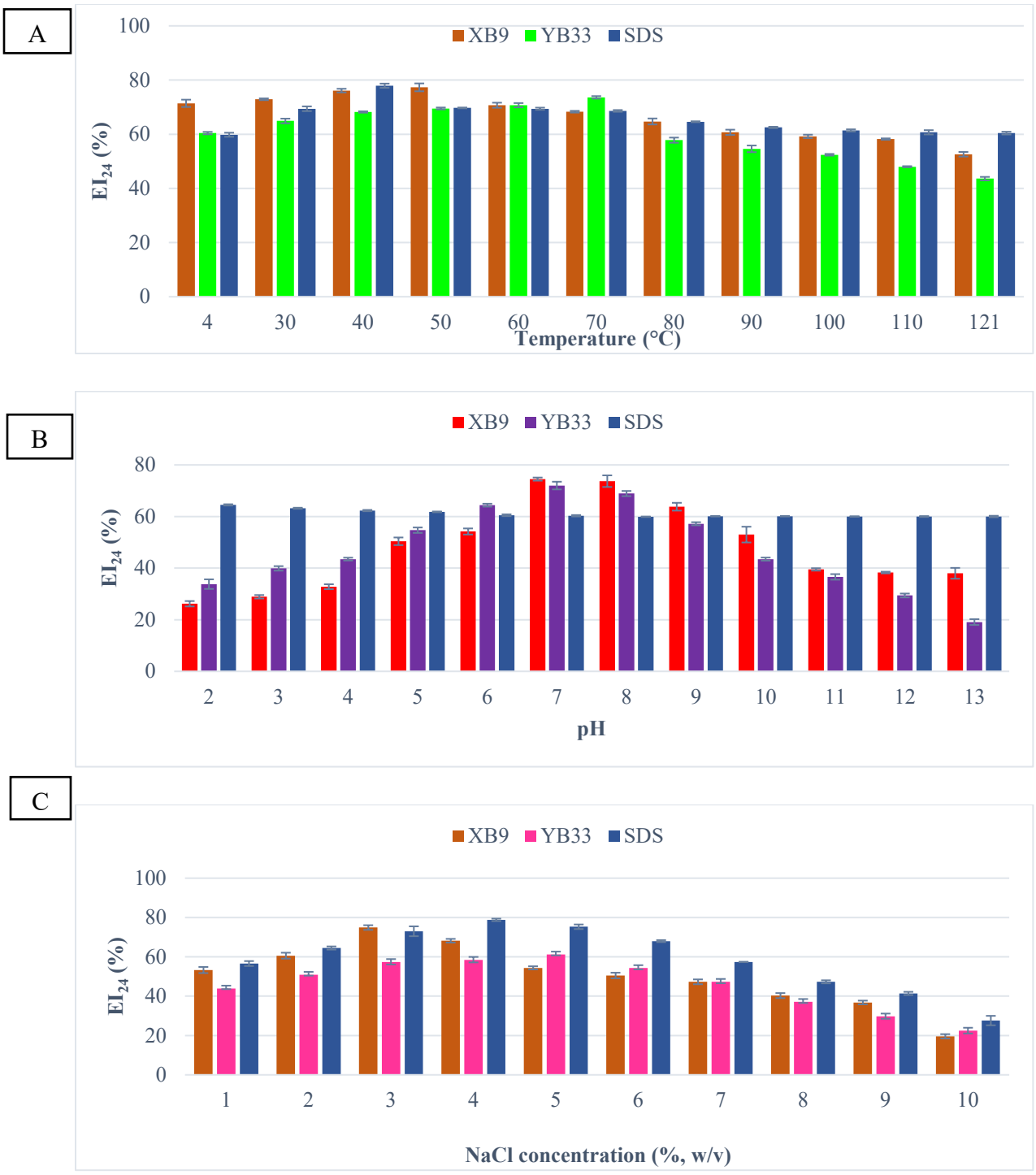

Fig. 5 Emulsifying activity of bioemulsifier XB9 and YB33 on sunflower oil at different: (A) temperatures (B) pH and (C) salinity. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Conclusions

In the present study, glycoprotein bioemulsifier production from two indigenous *Acinetobacter* sp. isolated from lipid-rich wastewater was reported in the presence of olive oil as inducible substrate, thus contributing to the reduction of process cost. The bioemulsifiers demonstrated high emulsifying activity in the presence of edible oils including sunflower oil, canola oil, castor oil and rice bran oil, as well as stability at extreme temperature, moderate salinity and pH. Hence, these bioemulsifiers (XB9 and YB33) represent a good candidate for various industrial and environmental applications, especially in the remediation of lipid-rich wastewater and enhanced-oil recovery.

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Conflict of interests

The authors declare that there are no conflicts of interest regarding the publication of this paper

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CHAPTER SIX

OPTIMIZATION OF BIOPROCESS PARAMETERS FOR ENHANCED PROTEASE PRODUCTION BY *BACILLUS ARYABHATTAI* AB15-ES USING RESPONSE SURFACE METHODOLOGY

This chapter has been submitted in the current format to the 'Bioprocess and Biosystems Engineering'

Optimization of bioprocess parameters for enhanced protease production by *Bacillus aryabhattai* Ab15-ES using response surface methodology

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Abstract Microbial proteases are the largest and complex group of enzymes that catalyze the hydrolysis of proteins by cleavage of peptide bonds that exist between amino acid residues in a polypeptide chain. They constitute more than 40% of the total worldwide production of enzymes, and their production is greatly influenced by fermentation media composition in addition to culture conditions such as pH, temperature and inoculum volume. Hence, response surface methodology (RSM) was applied for the optimization of bioprocess parameters for enhanced extracellular protease production by an indigenous protease-producing *Bacillus aryabhattai* Ab15-ES. "One-variable at-a-time" approach was employed for the selection of best carbon and nitrogen sources for maximum protease production. The optimum conditions for extracellular protease production obtained from quadratic model of RSM were temperature 40 °C, pH 7.8, inoculum volume 2.5% (v/v), maltose 12.35 g/L and beef extract 5.30 g/L. The coincidence of observed protease production (247.84 ± 2.31 U/mL) with predicted protease yield (248.19 U/mL) coupled with high coefficient of determination ($R^2 = 0.9880$, $P < 0.01$) confirmed the validity of the model. A 4.4-fold increase in protease production was obtained in the optimized medium using the basal medium as a reference. The present study is the first report on protease production and optimization from *Bacillus aryabhattai* using response surface methodology. These findings suggest a rational choice of optimized process conditions for commercial production of protease by *Bacillus aryabhattai* Ab15-ES for various biotechnological applications.

Keywords Protease; Bioprocess parameters; *Bacillus aryabhattai*; Response surface methodology; Lipid-rich wastewater; Optimization

Introduction

Proteases are the largest and most important group of enzymes constituting more than 60% of the total industrial enzyme market [1, 2]. They play a vital role in industrial biotechnology, especially in detergent, food, leather, and textile industries; pharmaceutical, organic chemical synthesis and wastewater treatment [3]. Proteases are ubiquitous in nature, and are produced by plants, animals and microbes. However, proteases of microbial origin are mostly preferred due to their broad biochemical diversity, ease of genetic manipulation, feasibility of mass culture, limited space for growth with high productivity, longer shelf life and less stringent storage requirements [4]. A wide range of microorganisms including bacteria, fungi, and yeasts produces proteases. However, mass production of proteases for commercial applications has been recorded among bacteria mainly in the genus *Bacillus* [5, 6], including *Bacillus clausii* [7], *Bacillus cereus* [8], *Bacillus circulans* [9], *Bacillus lehensis* [10], *Bacillus licheniformis* [6], *Bacillus megaterium* [11], *Bacillus mojavensis* [12], *Bacillus sterothermophilus* [13], *Bacillus pumilus* [14] and *Bacillus subtilis* [15].

In view of the growing trends of proteases which has prompted expansion of their uses, the overall costs of the enzyme production from downstream purification still formed the ultimate challenge to successful white biotechnology [16]. Optimization of fermentation conditions is the most important step for improved and cost-effective bioprocesses [17, 18, 49]. Conventional methods involving change of 'one-variable-at-a-time' is frequently used for the optimization of process parameters to achieve optimum cell density and high productivity of microbial metabolite. However, these approaches are time-consuming and thus expensive for simultaneous consideration of many variables [19]. In addition, they are laborious, require more experimental data and unable to detect interactions and optimal conditions among variables [20, 21].

Due to these drawbacks, statistical experimental designs have been recognized as ideal ways for bioprocess optimization studies [22, 23]. Statistical optimization by response surface methodology (RSM) is an efficient experimental strategy to maintain optimal conditions in a multi-variable system [16, 24]. RSM is an assemblage of mathematical and statistical techniques for modelling and analysis in applications where a response of interest is influenced by several variables and the objective is to optimize this response [7]. This method helps to identify effective factors, study interactions, select optimum conditions and quantify the relationships between one or more measured responses and the vital input factors in limited number of experiments [22, 25, 26]. Several fermentation processes have been optimized using this methodology [27, 28]. Central composite design (CCD) is used to estimate the coefficients of a quadratic model, and it has three groups of design points: two-level factorial or fractional factorial, axial and central points.

Protease optimization studies by CCD taking cognizance of fermentation medium composition and physical factors as objective functions have been reported [7, 17, 29, 30, 50]. It has been well established that

extracellular protease production by microorganisms is influenced by media composition, especially carbon and nitrogen sources in addition to culture conditions such as temperature, pH and inoculum volume [22, 31, 51]. Therefore, in the present study, optimization of process parameters was applied for enhanced production of protease by *Bacillus aryabhattai* Ab15-ES. "One-variable-at-a-time" approach was used for selection of best carbon and nitrogen sources. Thereafter, statistical optimization of the selected carbon and nitrogen sources together with temperature, pH and inoculum size for enhanced protease production was carried out using RSM.

Materials and methods

Chemicals

Azocasein used as substrate for protease assay was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

Sample collection and bacterial isolation procedures

Lipid-rich wastewater samples were collected from a poultry processing industry in Estcourt, KwaZulu-Natal (KZN) province, South Africa into sterile 500 mL Schott bottles. Samples (10 mL) were transferred to 250 mL Erlenmeyer flasks containing 100 mL mineral salt medium (MSM) consisting (in g/L): 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.2 $MgSO_4.7H_2O$, 0.02 $CaCl_2.2H_2O$ and 0.05 $FeCl_3.6H_2O$ supplemented with 2% olive oil, and then incubated at 37 ºC for 48 h at 150 rpm [32]. Enriched cultures (5 mL) were subsequently transferred into the second flask and cultivated under the same conditions. This process was repeated four times. Samples (0.1 mL) of the last enrichment cultures were serially diluted, plated onto nutrient agar (NA) and incubated at 37ºC for 24 h. Pure cultures obtained by sub-culturing on fresh NA plates were preserved at -80 ºC in a storage medium (500 µl of 80% sterile glycerol and 500 µL culture suspension).

Screening of the bacterial isolate for proteolytic activity

A loopful of bacterial culture was inoculated into 10 mL sterile nutrient broth and then incubated at 150 rpm (37 ºC) for 24 h. Proteolytic activity of the bacterial isolate was detected by addition of 20 µL of obtained culture supernatant into wells bored on skim milk agar plate [33]. Sterile nutrient broth was used as a control. Plates were incubated at 37 ºC for 48 h and observed for the formation of clear zones of casein hydrolysis, indicative of proteolytic activity. All experiments were carried out in triplicate.

Identification and phylogenetic analysis of the bacterial isolate

Genomic DNA extraction

Genomic DNA was isolated from bacterial culture grown overnight on NA plate at 37 °C using boiling method described by [34] with modifications. Freshly grown colonies (5-7) were suspended in 70 µL sterile distilled water, boiled at 100 ºC for 10 min and then cooled on ice for 5 min. This was centrifuged at 13000 rpm in a micro-centrifuge 5415D (Eppendorf, Hamburg, Germany) for 5 min. Cell-free supernatant was transferred into fresh sterile Eppendorf tubes and used as template DNA for the PCR assay.

Amplification, sequencing and analysis of 16S rRNA gene

Amplification of the 16S rRNA gene was carried out using universal bacterial primers F-5' CAGGCCTAACACATGCAAGTC-3' and R-5' GGGCGGTGTGTACAAGGC-3' [35]. The PCR mixture consisted of 1× buffer, 1mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 µm of each primer, 2 U of *Taq* polymerase and 2 µL template DNA in a total volume of 25 µL. The mixture was subjected to amplification using a thermocycler (Bio-Rad T100, Singapore) at initial denaturation of 95 ºC for 5 min, followed by 30 cycles of; denaturation at 95 ºC for 1 min, annealing at 55 ºC for 1 min, extension at 72 ºC for 1min, 30 sec and final extension at 72 ºC for 5 min. Amplification products were observed by electrophoresis in a 1.5% (w/v) agarose gel at 100 V for 45 min in a 1% TAE buffer. After staining in 1 mg/mL ethidium bromide for 15 min, products were viewed by UV illumination (Syngene, UK), and then sequenced (inqaba biotech, Pretoria, South Africa). The resulting sequences were compared with sequences in the GenBank database of the National Centre for Biotechnology Information (NCBI) using the Nucleotide Basic Local Alignment Search Tool (BLAST N) program (www.ncbi.n1m.nih.gov/BLAST). The sequences were aligned using the multiple sequences alignment tool, Clustal W. Phylogenetic tree was constructed with Molecular Evolutionary Genetics analysis (MEGA) version 6.0 software [36] using a neighbor-joining method.

Time course profile of growth and extracellular protease production

Basal medium used for growth and protease production profiling consisted of (in g/L): glucose, 10; peptone, 5; NaCl, 0.5; $CaCl_2$. $2H_2O$, 0.1; K_2HPO_4, 0.3; KH_2PO_4, 0.4 and $MgSO_4$. $7H_2O$, 0.1. The pH of the medium was adjusted to 7.2 with 1N HCl or 1N NaOH. One milliliter of the bacterial culture (OD_{600nm} = 1)) was inoculated into 99 mL basal medium in 250 mL Erlenmeyer flasks. The flasks were incubated at 150 rpm (37 °C) for 72 h in a shaking incubator. Samples were taken at 12-h intervals to determine bacterial growth and protease production. Bacterial growth was determined by measuring optical density at 600 nm using UV-Vis spectrophotometer (UVmini-1240, Schimadzu, Australia). Thereafter, samples were centrifuged at

10000 rpm for 10 min at 4 ºC using high-speed refrigerated centrifuge (Avanti J-26 XP, Beckman Coulter, USA). The obtained cell-free culture supernatants were then assayed for protease activity. All experiments were done in triplicate. Protease activity was determined according to the modified method of Secades and Guijarro [37] using azocasein as a substrate. One unit (U) of enzyme activity was defined as the amount of enzyme which yielded an increase in absorbance (440 nm) of 0.01 per minute under defined assay conditions.

Optimization of nutritional parameters for protease production

Preliminary screening of different carbon and nitrogen sources for maximum protease production was carried out using a "one-variable-at-a-time" approach. Simple and complex carbon sources such as fructose, sucrose, lactose, maltose, galactose, mannose, soluble starch as well as inorganic and organic nitrogen sources including NH_4Cl, KNO_3, yeast extract, skim milk, beef extract, malt extract, and soybean meal were supplemented separately in the production medium for extracellular protease production. The carbon and nitrogen sources were added at a concentration of 10 (g/L) and 5 (g/L), respectively. Homogenous bacterial culture [1% (v/v) of $OD_{600nm} = 1$] was inoculated into the production medium, and then incubated on a shaking incubator at 150 rpm for 48 h at 37 ºC. The fermentation medium was centrifuged at 10000 rpm at 4 ºC for 10 min. The obtained cell-free supernatant was assayed for protease activity, as previously described. All experiments were carried out in triplicate.

Statistical optimization of protease production

Response surface methodology

Response surface methodology (RSM) was used for the optimization of five independent variables including; maltose, beef extract, temperature, pH and inoculum volume. In this experiment, face centered CCD (FCCCD) was employed to determine optimum concentration of these variables for extracellular protease production. A total of 50 experimental runs were generated using Design-Expert 10 (Stat Ease Inc., Minneapolis, USA). Each numeric factor was varied at three different levels (-1, 0, +1). The central coded value of the variables was considered as zero. The minimum and maximum ranges of variables studied, and the full experimental plan with respect to their values in actual and coded levels are listed in Table 1.

Table 1 Experimental range of five independent variables used in FCCCD in terms of actual and coded levels

	Range of levels		
Variable	-1	0	+1
X_1: Temperature	30	35	40
X_2: pH	6	7	8
X_3: Inoculum volume	1	2	3
X_4: Maltose	5	10	15
X_5: Beef extract	2.5	5	7.5

The response values (Y) in each run were the average of the triplicate. The data obtained from the RSM on protease production were subjected to analysis of variance (ANOVA). The experimental results were fitted with the response surface regression procedure using the second order polynomial equation shown in eq. (1):

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response; β_o is the intercept term; $X_i X_j$ are independent variables, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient.

Results and discussion

Isolation, screening and identification of protease-producing bacteria

Among the bacterial strains isolated from the poultry processing wastewater, strain Ab15-ES was found to exhibit a large clear zone of casein hydrolysis after 48 h, suggesting high proteolytic activity of the bacterial strain. Such high extracellular enzymatic activity could largely be due to high rates of substrate utilization and hydrolysis by the bacterial isolate. Analysis of the 16S rRNA gene sequence of strain Ab15-ES showed that it exhibits close homology (100%) to *Bacillus aryabhattai* (KU556298.1). This study strain was therefore designated as *Bacillus aryabhattai* Ab15-ES. The relatedness of this strain to other protease-producing *Bacillus* sp., especially *Bacillus subtilis* VV reported to be a high alkaline protease-producing bacterial strain [38] is illustrated in the constructed phylogenetic tree (Fig. 1).

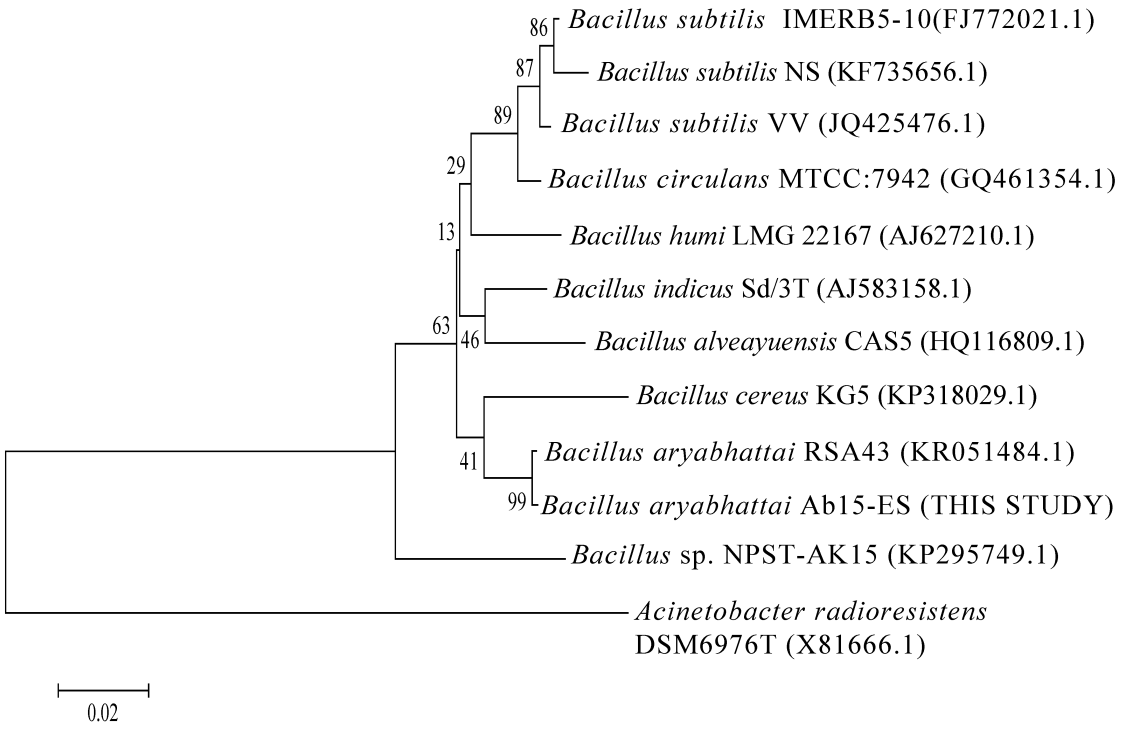


Fig. 1 Phylogenetic neighbor-joining tree based on 16S rRNA gene sequences of *Bacillus aryabhattai* Ab15-ES showing its relatedness to other protease-producing *Bacillus* strains. GenBank accession numbers are included in parentheses. Number at branching nodes are bootstrap percentages (based on 1000 replications). *Acinetobacter radioresistens* DSM6976T was used as an outgroup. Bar, 2% sequence divergence.

Growth kinetics and extracellular protease production

Bacillus aryabhattai Ab15-ES was cultivated in a basal medium for extracellular protease production by submerged fermentation. Growth of this strain was monitored at 12-h intervals in order to establish the exact phase at which optimum protease production was attained. The cell growth and protease production profile is presented in Fig. 2. Protease production was found to be growth-dependent, as parallel relationship was observed between cell growth and protease activity. Maximum protease production (56.42 U/mL) was recorded at 48 h during late exponential growth phase. Optimum protease production from *Bacillus licheniformis* N-2 [39] and *Bacillus subtilis* SHS-04 [40] during late exponential phase has been reported.

Beyond 48 h, a rapid decline in protease production was recorded. This may be due to inadequate availability of nutrients in the growth media, or autolysis resulting from build-up of the enzyme in the production media [39].

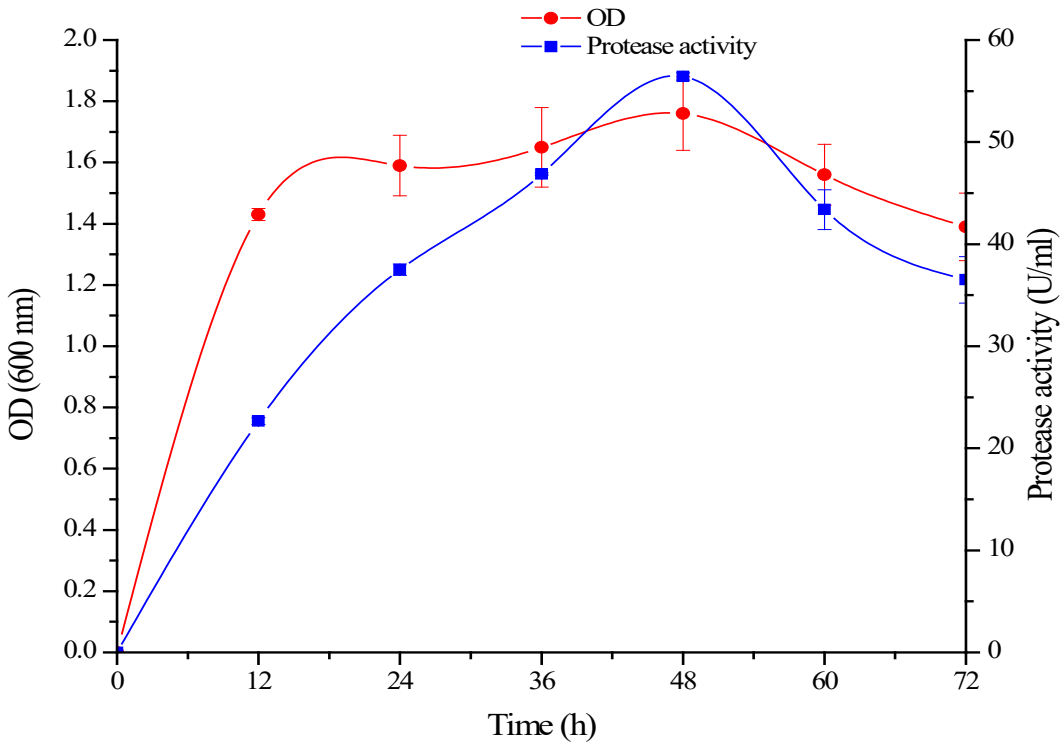


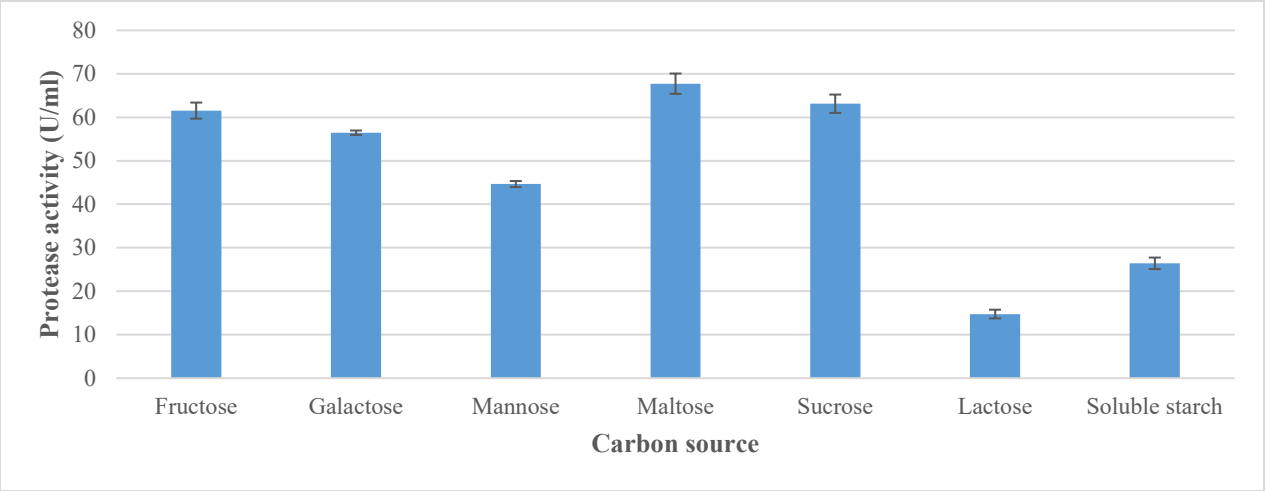
Fig. 2 Time course profile of growth and protease production by *Bacillus aryabhattai* Ab15-ES cultivated in a basal medium at 37 °C and 150 rpm during 72 h incubation. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Selection of best carbon and nitrogen sources for protease production

Preliminary screening of various carbon and nitrogen sources for maximum protease production from *Bacillus aryabhattai* Ab15-ES was investigated using "one-variable-at-a-time" approach. Glucose in the basal medium with protease production of 56.42 U/mL was substituted with other carbon sources including fructose, sucrose, soluble starch, lactose, maltose, galactose or mannose. The results showed variation in extracellular protease production on addition of different carbon sources. Maximum protease production (67.73 U/mL) was found in the medium containing maltose followed by sucrose and fructose (Fig. 3A). This supports the findings of [41] and [42] where highest protease yields were attained in a fermentation medium consisting of maltose as a carbon source.

Influence of organic and inorganic nitrogen sources on protease production was studied using the fermentation medium consisting of maltose as carbon source. The results showed higher protease production in the presence of organic nitrogen sources than inorganic nitrogen sources. Beef extract was recorded as the best nitrogen source to support protease production (72.75 U/mL), followed by malt extract (68.09 U/mL) and skim milk (63.45 U/mL) (Fig. 3B). This may be due to the presence of multi-nutrient elements including free amino acids, essential fatty acids, carbohydrates, etc. in organic nitrogen sources, which induced the growth of the organism for maximum protease production [43]. This is in agreement with previous studies in literature [44, 45]. Therefore, maltose and beef extract were selected as best carbon and nitrogen sources, respectively for the design of RSM.

A



B

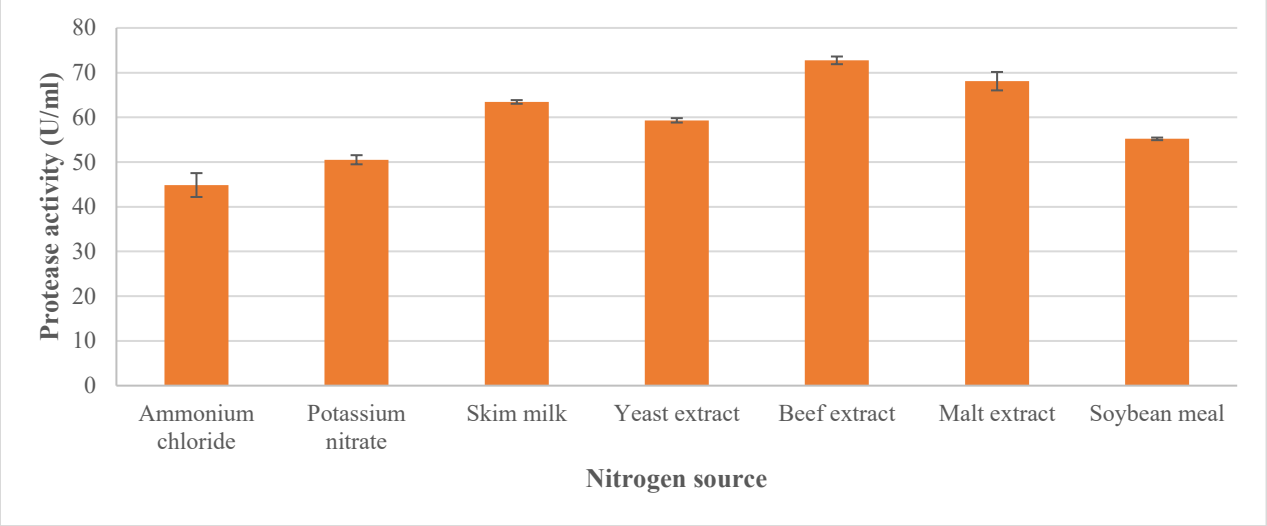


Fig. 3 Effect of various (A) carbon and (B) nitrogen sources on protease production by *Bacillus aryabhattai* Ab15-ES cultivated in a basal medium and incubated at 37 °C, 150 rpm for 48 h. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Optimization of bioprocess parameters for maximum protease production using RSM

Five independent variables including maltose, beef extract, temperature, pH and inoculum volume were optimized for enhanced protease production using FCCCD, and the results of predicted and observed responses of different runs are presented in Table 2. The mathematical model for protease production along with the independent variables is presented in the second-order polynomial equation illustrated in eq. (2)

$$Y = +98.87 + 5.48X_1 + 4.77X_2 + 6.01X_3 + 10.22X_4 + 8.43X_5 + 8.06X_1{*}X_2 + 7.76X_1{*}X_3 + 5.39X_1{*}X_4 + 6.87X_1{*}X_5 + 9.24X_2{*}X_3 + 6.03X_2{*}X_4 + 6.68X_2{*}X_5 + 5.01X_3{*}X_4 + 1.95X_3{*}X_5 + 8.53X_4{*}X_5 + 10.13X_1^2 + 0.93X_2^2 + 4.17X_3^3 + 11.13X_4^2 + 2.82X_5^2 \quad (2)$$

Where Y is the predicted protease activity, X_1 is temperature, X_2 is pH, X_3 is inoculum volume, X_4 is maltose, and X_5 is beef extract. The statistical significance of the second-order polynomial equation was assessed by ANOVA and data are shown in Table 3. Values of "Prob > F" less than 0.05 show the significant of the model terms. Values greater than 0.1000 indicate the model terms are not significant. In this study, X_1, X_2, X_3, X_4, and X_5 were significant model terms. The interaction effects of the parameters: $X_1{*}X_2$ ($P < 0.0001$), $X_1{*}X_3$ ($P < 0.0001$), $X_1{*}X_4$ ($P < 0.0001$), $X_1{*}X_5$ ($P < 0.0001$), $X_2{*}X_3$ ($P < 0.0001$), $X_2{*}X_4$ ($P < 0.0001$), $X_2{*}X_5$ ($P < 0.0001$), $X_3{*}X_4$ ($P < 0.0001$), $X_3{*}X_5$ ($P = 0.0139$), $X_4{*}X_5$ ($P < 0.0001$) were also significant ($P < 0.05$). The F-value of 90.48 and very low probability F-value (< 0.0001) coupled with non-significant lack of fit ($P = 0.0685$) indicate that the model was significant. This also implied that, the model equation was adequate for predicting protease production under any interaction of values of the parameters.

Table 2 FCCCD of five independent variables with predicted and observed protease production

Std	Run	X_1: Temperature	X_2: pH	X_3: Inoculum volume	X_4: Maltose	X_5: Beef extract	Observed response (U/mL)	Predicted response (U/mL)
23	1	30	8	3	5	7.5	118.8	117.5
12	2	40	8	1	15	2.5	114.6	115.9
28	3	40	8	1	15	7.5	169.5	168.2
22	4	40	6	3	5	7.5	112.9	110.7
1	5	30	6	1	5	2.5	163.6	162.0
42	6	35	7	2	10	7.5	109.4	110.0
40	7	35	7	2	15	5	119.1	117.6
32	8	40	8	3	15	7.5	234.6	233.0
10	9	40	6	1	15	2.5	105.2	104.3
49	10	35	7	2	10	5	94.4	92.6
17	11	30	6	1	5	7.5	128.7	126.3
37	12	35	7	1	10	5	93.4	95.08
39	13	35	7	2	5	5	103.3	104.7
36	14	35	8	2	10	5	103.1	107.6
47	15	35	7	2	10	5	102.9	100.4
5	16	30	6	3	5	2.5	116.3	118.1
16	17	40	8	3	15	2.5	159.5	156.2
21	18	30	6	3	5	7.5	96.8	99.79
45	19	35	7	2	10	5	101.3	99.71
6	20	40	6	3	5	2.5	111.6	110.5
25	21	30	6	1	15	7.5	133.5	131.7
34	22	40	7	2	10	5	115.3	113.8
27	23	30	8	1	15	7.5	132.5	134.6
41	24	35	7	2	10	2.5	94.7	95.69
43	25	35	7	2	10	5	97.3	98.1
50	26	35	7	2	10	5	97.3	98.1
48	27	35	7	2	10	5	97.3	98.1
14	28	40	6	3	15	2.5	121.4	119.5
2	29	40	6	1	5	2.5	115.1	110.0
19	30	30	8	1	5	7.5	106.3	104.1
20	31	40	8	1	5	7.5	118.2	116.5
3	32	30	8	1	5	2.5	102.6	104.4
38	33	35	7	3	10	5	116.9	113.3
9	34	30	6	1	15	2.5	133.4	131.3
4	35	40	8	1	5	2.5	97.6	96.68
46	36	35	7	2	10	5	97.3	98.1
44	37	35	7	2	10	5	97.3	98.1
7	38	30	8	3	5	2.5	104.3	106.2
31	39	30	8	3	15	7.5	158.6	157.2
18	40	40	6	1	5	7.5	115.4	113.6
35	41	35	6	2	10	5	95.2	94.3
15	42	30	8	3	15	2.5	128.1	127.6
13	43	30	6	3	15	2.5	117.3	116.1
8	44	40	8	3	5	2.5	136.4	132.3
33	45	30	7	2	10	5	109.7	106.5
26	46	40	6	1	15	7.5	138.6	134.7
11	47	30	8	1	15	2.5	104.8	100.1
24	48	40	8	3	5	7.5	155.6	151.4
29	49	30	6	3	15	7.5	125.7	120.9
30	50	40	6	3	15	7.5	164.8	163.4

Table 3 Analysis of variance (ANOVA) for the response surface quadratic model for optimization of protease production

Source	SS	df	MS	*F*-value	*P* value
Model	32072.95	20	32072.95	90.48	< 0.0001[a]
X_1	1020.70	1	1020.70	57.59	< 0.0001[a]
X_2	774.65	1	774.65	43.71	< 0.0001[a]
X_3	1226.76	1	1226.76	69.22	< 0.0001[a]
X_4	3552.27	1	3552.27	200.43	< 0.0001[a]
X_5	2417.89	1	2417.89	136.42	< 0.0001[a]
X_1*X_2	2079.64	1	2079.64	117.34	< 0.0001[a]
X_1*X_3	1925.57	1	1925.57	108.65	< 0.0001[a]
X_1*X_4	928.91	1	928.91	52.41	< 0.0001[a]
X_1*X_5	1510.16	1	1510.16	85.21	< 0.0001[a]
X_2*X_3	2734.86	1	2734.86	154.31	< 0.0001[a]
X_2*X_4	1161.74	1	1161.74	65.55	< 0.0001[a]
X_2*X_5	1428.85	1	1428.85	80.62	< 0.0001[a]
X_3*X_4	801.90	1	801.90	45.25	< 0.0001[a]
X_3*X_5	121.56	1	121.56	6.86	0.0139[a]
X_4*X_5	2328.86	1	2328.86	131.40	< 0.0001[a]
X_1^2	253.61	1	253.61	14.31	0.0007[a]
X_2^2	2.12	1	2.12	0.12	0.7319
X_3^2	42.93	1	42.93	2.42	0.1305
X_4^2	306.18	1	306.18	17.28	0.0003[a]
X_5^2	19.68	1	19.68	1.11	0.3006
Residual	513.98	29			
Lack of fit	465.05	22	21.14	3.02	0.0685
Pure error	48.93	7	6.99		
Cor. Total	32586.93	49			

SS: Sum of squares; df: degree of freedom; MS: Mean square; *F*: *F* ratio; *P*: probability value; $R^2 = 0.9880$; Adj. $R^2 = 0.9797$; Pred. $R^2 = 0.9615$; Adeq. precision = 49.56; [a], statistically significant ($P < 0.05$)

The lack of fit *F*-value of 3.02 implies that there was a 6.85% chance that a lack of fit *F*-value this large could occur due to noise. The coefficient of determination (R^2) is an index of the degree of variation in the observed response values that can be explained by the experimental parameters and their combinations. The closer the value of R^2 to 1, the better the correlation between observed and predicted values, and the better the reliability of the model. The R^2 of the polynomial equation was found to be 0.9880 indicating that 98.80% of the variability in the yield (protease production) could be explained by the model and only about 1.20% of the total variation cannot be explained by the model. The predicted R^2 value of 0.9615 was in reasonable agreement with adjusted R^2 value of 0.9797. This implied a good adjustment between the observed and predicted values. "Adequate precision" measures the signal to noise ratio. A ratio greater than 4 is desirable for a model to be a good fit. Our ratio of 49.56 indicates an adequate signal. This model can be used to navigate the design space. In addition, coefficient of variation, standard deviation, mean and

predicted residual sum of squares values of 3.55%, 4.21, 118.70 and 1801.44, respectively were obtained from the model.

The high degree of similarity between the predicted and observed responses is showed by the actual versus predicted plot (Fig. 4). In this case, even distribution of the points around the diagonal signifies a good fit of the model.

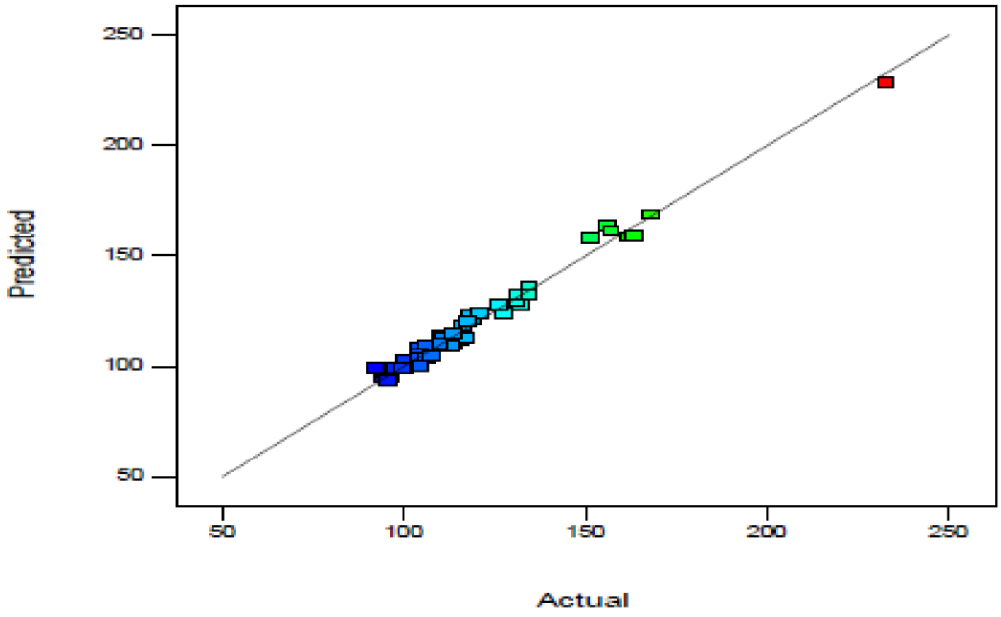


Fig. 4 Predicted versus actual response plot for protease production by *Bacillus aryabhattai* Ab15-ES.

For proper understanding of the interaction effects and optimum values of combination of the five independent variables for maximum protease production, contour plots were generated and are presented in Fig. 5A-J. These plots were constructed from a pair-wise interaction of the independent parameters, while keeping other parameters constant at their center point level using a Design Expert 10 software. The elliptical contour plots indicate a significant interaction among variables while a circular contour plot implies a negligible interaction [46,47]. From the contour plot, significant interaction was notable among the parameters, as in case of temperature and inoculum volume.

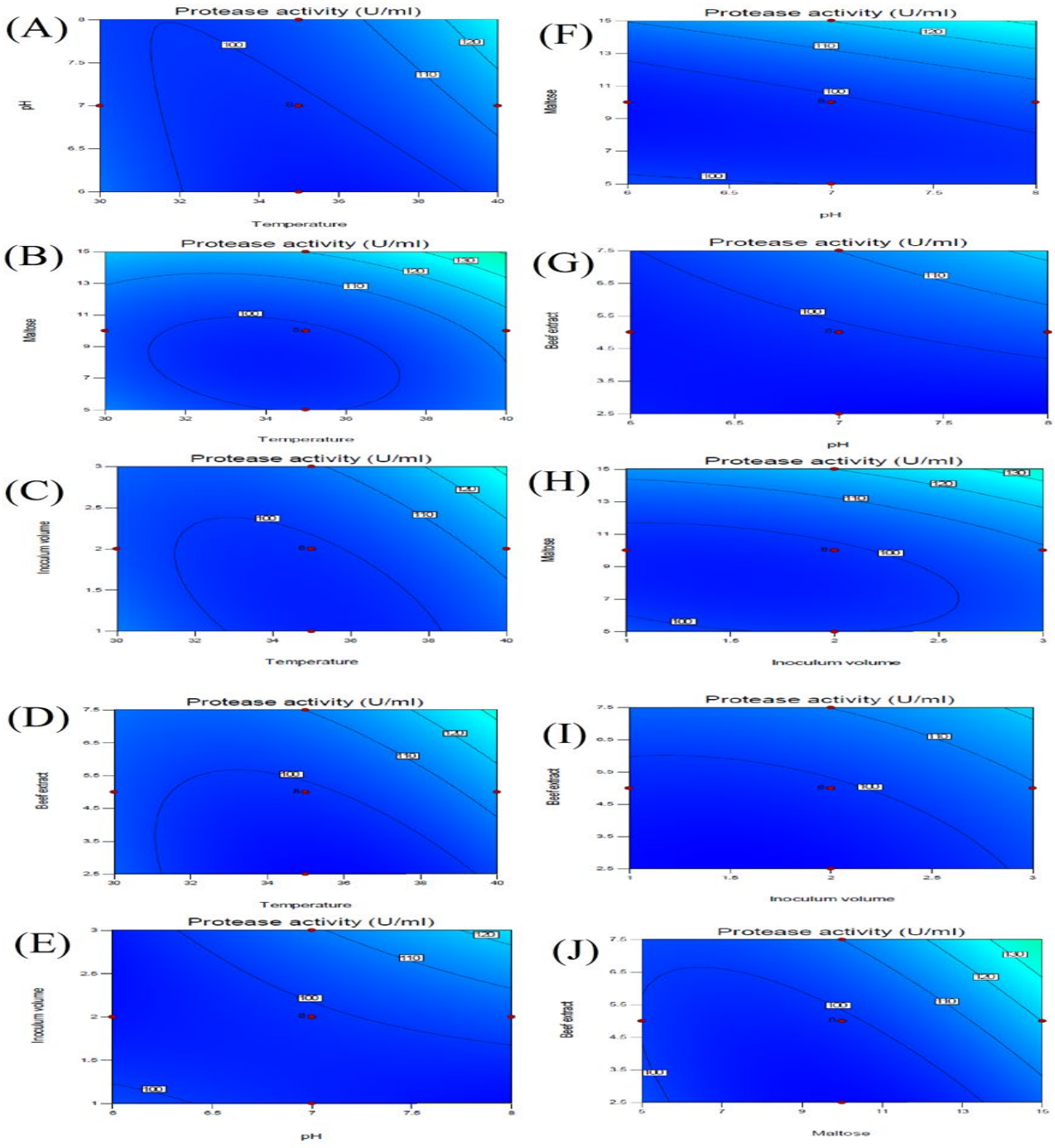


Fig. 5 Contour plot of protease production by *Bacillus aryabhattai* Ab15-ES showing interaction between (A) temperature and pH, (B) maltose and temperature, (C) inoculum volume and temperature, (D) beef extract and temperature, (E) inoculum volume and pH, (F) maltose and pH, (G) beef extract and pH, (H) maltose and inoculum volume, (I) beef extract and inoculum volume, and (J) beef extract and maltose.

Experimental validation of the optimized process conditions

The predicted results by the model showed that maximum protease production could be achieved when optimum temperature, pH, inoculum volume, maltose and beef extract were established at 40 ºC, 7.8, 2.5% (v/v), 12.35 g/L and 5.30 g/L, respectively. Under these conditions, maximum predicted value of protease production was 248.19 U/mL. This optimization result was further validated using the predicted optimum conditions, resulting in maximum mean protease production of 247.84 ± 2.31 U/mL at 48 h during late log phase (Fig. 6), found to be almost equal to the predictive yield. A significant 4.4-fold increase in protease production by *Bacillus aryabhattai* Ab15-ES was obtained after optimization of the bioprocess parameters using the basal medium as reference. The need for optimization of culture conditions for maximum protease production is imperative, since microorganisms possess specific physicochemical and nutritional requirements for growth and enzyme production [18]. Enhancement of protease production from *Bacillus aryabhattai* Ab15-ES by response surface methodology was found to be higher than that of *Bacillus* sp. HTS102 [30] and *Brevibacterium linens* DSM 20158 [48].

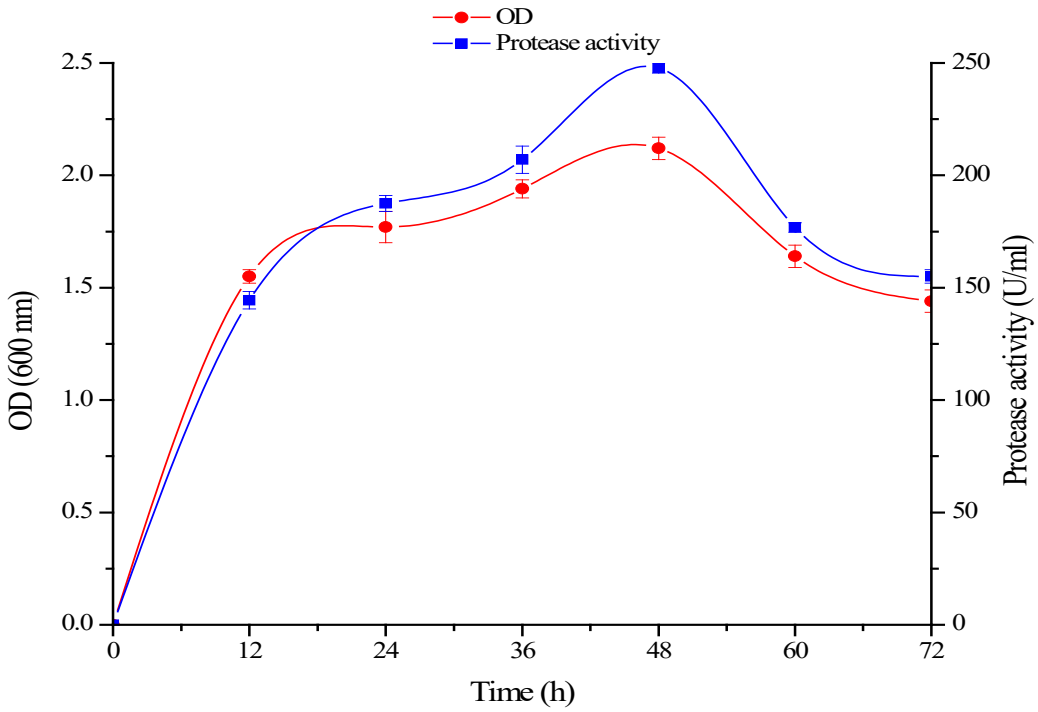


Fig. 6 Time course profile of growth and protease production by *Bacillus aryabhattai* Ab15-ES under the predicted optimal conditions. The culture medium consisted of maltose 12.35 g/L, beef extract 5.30 g/L, inoculum volume 2.5% (v/v) with initial pH adjusted to 7.8, and incubated at 40 °C. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Conclusions

In the present study, FCCCD was used for the optimization of bioprocess parameters for maximum protease production from *Bacillus aryabhattai* Ab15-ES using submerged fermentation process. Protease production depends mainly on temperature, pH, inoculum volume, maltose and beef extract. The observed protease production value agreed with that of predicted protease production, thus confirming the validity of the model. Under these optimum conditions, a 4.4-fold increase in protease production was obtained using the basal medium as reference. These findings suggest that *Bacillus aryabhattai* Ab15-ES can be used for mass production of protease for various biotechnological applications, thus minimizing overall costs of the enzyme production.

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CHAPTER SEVEN

OPTIMIZATION OF CULTURE CONDITIONS FOR ENHANCED LIPASE PRODUCTION BY AN INDIGENOUS *BACILLUS ARYABHATTAI* SE3-PB USING RESPONSE SURFACE METHODOLOGY

This chapter has been submitted in the current format to the 'Biotechnology and Biotechnological Equipment'

Optimization of culture conditions for enhanced lipase production by an indigenous *Bacillus aryabhattai* SE3-PB using response surface methodology

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ABSTRACT

Lipases are enzymes that catalyze the hydrolysis of fats into fatty acids and glycerol at the water-lipid interface as well as involved in a range of bioconversion reactions in non-aqueous and micro-aqueous environments. In this present study, optimization of culture conditions for extracellular lipase production by an indigenous lipase-producing bacterial strain isolated from lipid-rich wastewater was carried out using response surface methodology. The study isolate was identified as *Bacillus aryabhattai* SE3-PB by PCR amplification and analysis of 16S rDNA. Sunflower oil was found to induce maximum lipase production. Face centered central composite design revealed that temperature (40 °C), pH (7.6), inoculum volume (2.8%, v/v), agitation (193 rpm) and inducer oil concentration (2%, v/v) were optimum culture conditions for maximum lipase production. The observed lipase production (264.02 ± 1.94 U/ml) found to be in agreement with predicted lipase yield (265.82 U/ml) coupled with high coefficient of determination (R^2 = 0.9919, $P < 0.01$) confirmed the validity of the model. A 7.2-fold increase in lipase production was obtained in the optimized medium relative to the basal medium. These findings provide first report on production and optimization of lipase by *Bacillus aryabhattai* SE3-PB, and suggest a rational choice of optimum process conditions for commercial production of lipase by *Bacillus aryabhattai* SE3-PB for various biotechnological applications, especially in the remediation of lipid-rich wastewater.

KEYWORDS: Lipase; *Bacillus aryabhattai* SE3-PB; lipid-rich wastewater; face centered central composite design; response surface methodology

Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that catalyze the hydrolysis of lipids into di-acylglycerides, mono-acylglycerides, glycerol and fatty acids over an oil-water interface [1-3]. In addition, they are involved in a variety of bioconversion reactions including esterification, interesterification, transesterification, alcoholysis, acidolysis and aminolysis in non-aqueous and micro-aqueous milieu [4-6]. Because of their versatility, lipases are employed as enzyme of choice for numerous biotechnological applications including food, leather, cosmetic, detergent, paper and textile industries as well as in pharmaceutical and treatment of fat-containing waste effluents [7-9].

Lipases are ubiquitous in nature and are produced by plants, animals, and microorganisms [4,10]. However, microbial lipases formed the most widely used class of enzymes due to their selectivity, lower production cost, ease of genetic manipulation, high productivity, regiospecificity, stereospecificity, broad substrate specificity, and ability to catalyze heterogeneous reactions at the interface of water soluble and water insoluble systems [8,11-13]. Microorganisms such as bacteria, yeast, fungi and actinomycetes have been reported as potential producers of lipase [14-17]. They are found in different habitats including industrial wastes, vegetable oil processing industries, dairy industries, oil-contaminated sites, among others [18]. Lipase production from many species of *Bacillus* including *Bacillus pumilus* [19], *Bacillus subtilis* [20], *Bacillus thermoleovorans* [21], *Bacillus cereus* [22], *Bacillus coagulans* [23], *Bacillus megaterium* [24], and *Bacillus licheniformis* [25] has been reported. However, this is the first report on production and optimization of lipase from *Bacillus aryabhattai*.

The production of extracellular bacterial lipases is influenced by fermentation parameters such as temperature, pH, inoculum size, agitation, carbon sources, inducer sources and concentrations [26-28]. The search for optimal conditions of the above-mentioned parameters for lipase production in fermentation processes is imperative in order to obtain high yields of the enzyme at lower costs. A classical and conventional practice of achieving these is by ‘one variable-at-a-time approach’. This approach has been proved to be laborious, time-consuming and costly for simultaneous consideration of many variables in addition to its inability to depict interactive effects among factors and locate optimum conditions among variables. In order to overcome these drawbacks, statistical experimental designs by response surface methodology (RSM) have been recognized as an effective approach in many biotechnological processes [26,29].

Response surface methodology is a collection of mathematical and statistical techniques for designing experiments, modelling and searching for optimum conditions of factors influencing the responses. It is widely used to identify effective factors, study interactions, select optimum conditions and quantify the

relationships between one or more measured responses and the vital input factors in a limited number of experiments [11]. Furthermore, RSM has been successfully applied to evaluate and optimize the effect of process parameters in the production of lipase [28,30,31]. A central composite design (CCD) is the most commonly used response surface design when the experimental design is defined by the upper and lower limits of each parameter [32]. Several bioprocess parameters have been optimized for enhanced lipase production using CCD [15,16,33,34,].

The continuous demand for industrial lipase production with potential biotechnological applications necessitated the need to bioprospect for native and robust/hyperactive bacterial strains capable of mass production of this enzyme. In this study, optimization of culture conditions for enhanced extracellular lipase production by an indigenous *Bacillus aryabhattai* SE3-PB was investigated using RSM. Effect of various inducer oils on lipase production was also studied. Thereafter, optimization of the selected inducer concentrations along with other process parameters, including temperature, pH, agitation speed, and inoculum volume for enhanced lipase production was carried out using face centered CCD (FCCCD).

Materials and methods

Chemicals

p-nitrophenyl palmitate (*p*-NPP) used as substrate for lipase assay was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

Sample collection and bacterial isolation procedures

Lipid-rich wastewater samples were collected from an oil mill industry in Pietermaritzburg, KwaZulu-Natal (KZN) province, South Africa in sterile 500 ml Schott bottles. Samples (10 ml) were transferred to 250 ml Erlenmeyer flasks containing 100 ml mineral salt medium (MSM) consisting (in g/L): 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.2 $MgSO_4.7H_2O$, 0.02 $CaCl_2.2H_2O$ and 0.05 $FeCl_3.6H_2O$ supplemented with 1% olive oil, and then incubated at 37 ºC for 48 h at 130 rpm [35]. Enriched cultures (5 ml) were subsequently transferred into a second flask and cultivated under the same conditions. This process was repeated four times. Samples (0.1 ml) of the last enrichment cultures were serially diluted, plated onto nutrient agar (NA) and incubated at 37 ºC for 24 h. Pure cultures obtained by sub-culturing on fresh NA plates were preserved as glycerol stock at -80 ºC.

Screening of bacteria for lipolytic activity

Lipolytic activity of the bacteria was assessed by using solid media containing different lipid sources, such as olive oil and Tween-20. Enzymatic activity was detected by visual observation and measurement of clear zone on the agar surface.

Screening of bacteria for lipolytic activity using Tween-20 agar

Tween-20 agar consisting (in %, w/v, v/v): peptone 1, NaCl 0.5, $CaCl_2.2H_2O$ 0.01, agar 2 and Tween-20 1 was prepared as described by Gopinath *et al*. [36]. A loopful of bacterial culture was inoculated into 10 ml sterile nutrient broth and then incubated at 130 rpm (37 ºC) for 24 h. Twenty microliter of obtained culture supernatant was added into wells bored on Tween-20 agar plate, and then incubated at 37 ºC for 48 h. Sterile nutrient broth was used as a control. Visible precipitate of calcium salts around the wells resulting from formation of fatty acid from lipid hydrolysis indicates the presence of lipolytic activity. All experiments were carried out in triplicate.

Screening of bacteria for lipolytic activity using phenol red agar

Phenol red agar consisting (in %, w/v, v/v): phenol red dye 0.01, olive oil 0.1, $CaCl_2$ 0.1 and agar 2 was prepared as previously described [37]. The pH of the medium was adjusted to 7.3 using 1N NaOH or 1N HCl. Culture supernatant (20 µl) was added into the agar wells, and then incubated at 37 ºC for 48 h. The change in the color of phenol red dye to yellow was used as an indication of lipolytic activity. All experiments were carried out in triplicate.

Molecular identification of lipase-producing bacteria and phylogenetic analysis

Genomic DNA extraction

Genomic DNA was isolated from bacterial culture grown overnight on NA plate at 37°C using boiling method described by Akinbowale *et al*. [38] with modifications. Freshly grown colonies (5-7) were suspended in 70 µl sterile distilled water and then boiled at 100 ºC for 10 min before cooling on ice for 5 min. This was centrifuged at 13000 rpm in a micro-centrifuge 5415D (Eppendorf, Hamburg, Germany) for 5 min. Cell-free supernatant was transferred into fresh sterile Eppendorf tubes and used as template DNA for the PCR reaction.

Amplification, sequencing and analysis of 16S rRNA gene

Amplification of the 16S rDNA of the lipase-producing bacterial isolate was carried out using the universal bacterial primers F-5'-CAGGCCTAACACATGCAAGTC-3' and R-5'-GGGCGGTGTGTACAAGGC-3'

[39]. The PCR mixture consisted of 1× buffer, 1mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 µm of each primer, 2 U of *Taq* polymerase and 2 µl template DNA in a total volume of 25 µl. The mixture was subjected to amplification using a thermocycler (Bio-Rad T100, Singapore) at initial denaturation of 95 ºC for 5 min, followed by 30 cycles of; denaturation at 95 ºC for 1 min, annealing at 55 ºC for 1 min, extension at 72 ºC for 1min, 30 sec and final extension at 72 ºC for 5 min. Amplification products were observed by electrophoresis in a 1.5% (w/v) agarose gel at 100 V for 45 min in a 1% TAE buffer. After staining in 1 mg/ml ethidium bromide for 15 min, products were viewed by UV illumination (Syngene, UK), and then sequenced (inqaba biotech, Pretoria, South Africa). The resulting sequences were compared with sequences in the GenBank database of National Centre for Biotechnology Information (NCBI) using the Nucleotide Basic Local Alignment Search Tool (BLAST N) program (www.ncbi.n1m.nih.gov/BLAST). The sequences were aligned using the multiple sequences alignment tool, Clustal W. Phylogenetic tree was constructed with Molecular Evolutionary Genetics analysis (MEGA) version 6.0 software [40] using a neighbor-joining method.

Kinetics of growth and extracellular lipase production

The production medium composed (in %, w/v, v/v): peptone 0.5, yeast extract 0.5, NaCl 0.05, $CaCl_2$ 0.005, and olive oil 1.0 emulsified with gum arabic 0.5 [41]. The pH of the medium was adjusted to 7.2 with 1 N NaOH or 1 N HCl. One milliliter of the bacterial culture (OD_{600nm} = 1) was inoculated into 99 ml basal medium in 250 ml Erlenmeyer flasks. The flasks were then incubated at 37 ºC (130 rpm) for 72 h in a shaking incubator. Samples were taken at 12-h intervals to determine bacterial growth and lipase production. Bacterial growth was determined by measuring optical density at 600 nm using UV-Vis spectrophotometer (UVmini-1240, Schimadzu, Australia). Subsequently, samples were centrifuged at 10000 rpm for 10 min at 4 ºC using high-speed refrigerated centrifuge (Avanti J-26 XP, Beckman Coulter, USA). The obtained culture supernatants (crude enzyme) were then assayed for lipase activity. Lipase activity was determined as described by Winkler and Stuckmann [42] with some modifications using *p*-NPP as a substrate. One unit (U) of lipase activity was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol ml^{-1} min^{-1} under standard assay conditions. All experiments were done in triplicate.

Optimization of culture conditions for maximum lipase production

Effect of inducer oils on lipase production

Various vegetable oils including palm oil, castor oil, sunflower oil, canola oil or rice bran oil were screened for the ability to induce maximum lipase production by *Bacillus aryabhattai* SE3-PB. Olive oil in the fermentation medium for lipase production was replaced with (1%, v/v) of the tested oils while keeping the

remaining parameters constant. Homogenous bacterial culture [1% (v/v) of OD_{600nm} = 1] was inoculated into the production medium, and then incubated in a shaking incubator at 130 rpm for 48 h at 37 ºC. The fermentation medium was centrifuged at 10000 rpm at 4 ºC for 10 min. The obtained cell-free culture supernatant was assayed for lipase activity, as previously described. All experiments were carried out in triplicate.

Response surface methodology

Response surface methodology (RSM) was employed for the optimization of selected independent variables including inducer oil concentration (1-3%,v/v), agitation (100-200 rpm), pH (6-8), temperature (30-40 °C) and inoculum volume (1-3%,v/v) that greatly influence extracellular lipase production using FCCCD. These variables were tested at three coded levels (-1, 0, +1). The minimum and maximum ranges of variables studied, and the full experimental plan with respect to their values in actual and coded levels are listed in Table 1.

Table 1. Experimental range of independent variables used in FCCCD in terms of actual and coded levels

	Range of levels		
Variable	-1	0	+1
X_1: Temperature	30	35	40
X_2: pH	6	7	8
X_3: Inoculum volume	1	2	3
X_4: Agitation	100	150	200
X_5: Inducer oil concentration	1	2	3

A total of 50 experimental runs were generated using a statistical software package 'Design-Expert 10' (Stat Ease Inc., Minneapolis, USA). The average of the triplicate experiment in each run represent the response values (Y). The data obtained from the FCCCD for lipase production were subjected to analysis of variance (ANOVA) in order to assess the significance of the developed model. The experimental results were fitted with the response surface regression procedure using the following second order polynomial equation shown in eq. (1):

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response; β_o is the intercept term; $X_i X_j$ are independent variables, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient.

Results and discussion

Isolation, screening and identification of lipase-producing bacteria

Lipid-rich wastewater samples were collected from an edible oil mill industry in Pietermaritzburg, KwaZulu-Natal province of South Africa. The rational choice of this site was to provide a good environment for lipolytic organisms to grow. Samples were enriched in MSM supplemented with 1% (v/v) olive oil as a sole carbon source in order to stimulate the growth of lipolytic organisms [35]. Lipase-producing bacteria isolated from lipid-rich wastewater have been reported [43-46]. Among the isolated bacterial strains screened for extracellular lipase production on solid media consisting of Tween-20 as inducible substrate, strain SE3-PB was found to exhibit higher lipolytic activity. This was based on the formation of large visible calcium salt precipitates around the agar well, resulting from the breakdown of Tween-20 into fatty acids (Result not shown). Tween-20 is a commonly used substrate for detecting bacterial lipolytic activity, as it promotes optimal contact between cells and/or enzymes and substrate [47]. Further screening of strain SE3-PB for lipolytic activity was carried out using a chromogenic approach involving the use of phenol red dye, and olive oil as inducible substrate. Phenol red is a pH indicator with end point at pH 7.3-7.4. The release of fatty acids from lipolysis reaction of olive oil caused a decrease in the pH of the dye, resulting in yellow coloration, an indication of lipolytic activity of the tested bacterial strain [37]. Analysis of the 16S rRNA gene sequence of strain SE3-PB showed that it exhibits close homology (100%) to *Bacillus aryabhattai* (KX781238.1). This study strain was therefore designated as *Bacillus aryabhattai* SE3-PB. The relatedness of this strain to other lipase-producing *Bacillus* sp. is illustrated in the constructed phylogenetic tree (Figure 1).

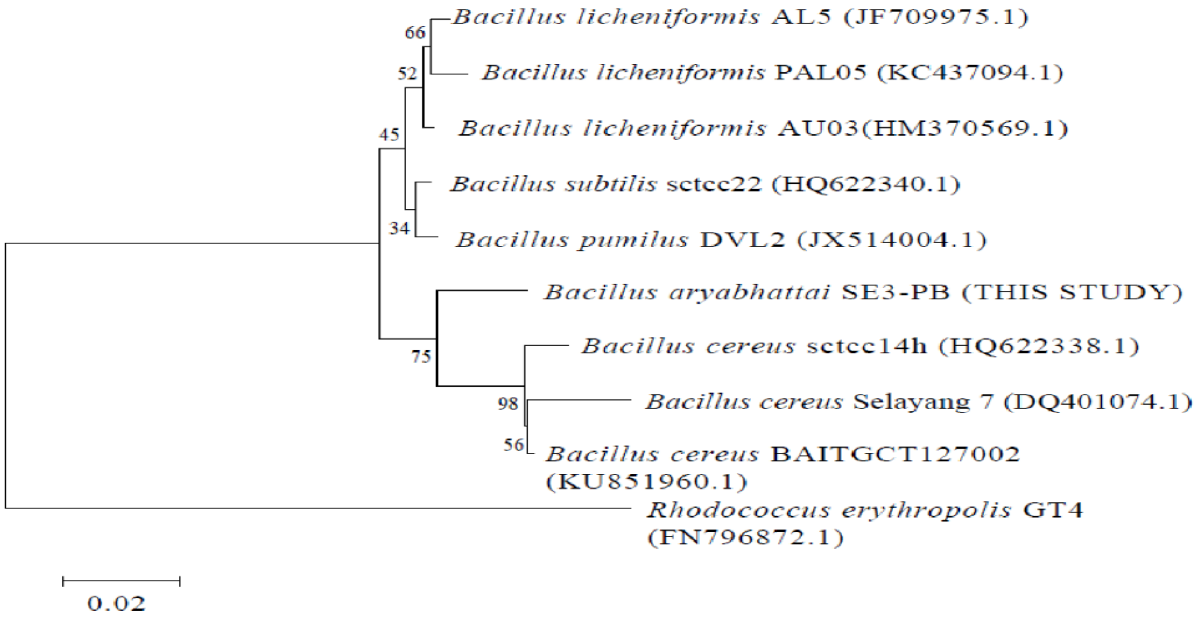

Figure 1. Phylogenetic neighbor-joining tree based on 16S rRNA gene sequences of *Bacillus aryabhattai* SE3-PB showing its relatedness to other lipase-producing *Bacillus* strains. GenBank accession numbers are included in parentheses. Number at branching nodes are bootstrap percentages (based on 1000 replicates). *Rhodococcus erythropolis* GT4 was used as an outgroup. Bar indicate 2% sequence divergence.

Kinetics of growth and lipase production

In order to ascertain the exact growth phase at which maximum extracellular lipase production was obtained, *Bacillus aryabhattai* SE3-PB was grown in a basal medium in shake flasks for 72 h. Culture samples were taken at 12-h intervals and assayed for bacterial growth and lipase production. Lipase production was found to be growth-linked, as parallel relationship was recorded between cell growth and lipase activity. Lipase production was initiated at early log phase and reached optimum during late exponential growth phase. Maximum lipase production (36.75 U/ml) was recorded at 48 h of cultivation period (Figure 2). This is similar to the findings of Jagtap *et al*. [48] and Bacha *et al*. [49] in which optimum lipase production by *Acinetobacter haemolyticus and Bacillus stearothermophilus* was recorded during late exponential phase. Beyond 48 h, there was a decrease in lipase activity, probably due to the presence of proteases in the fermentation medium.

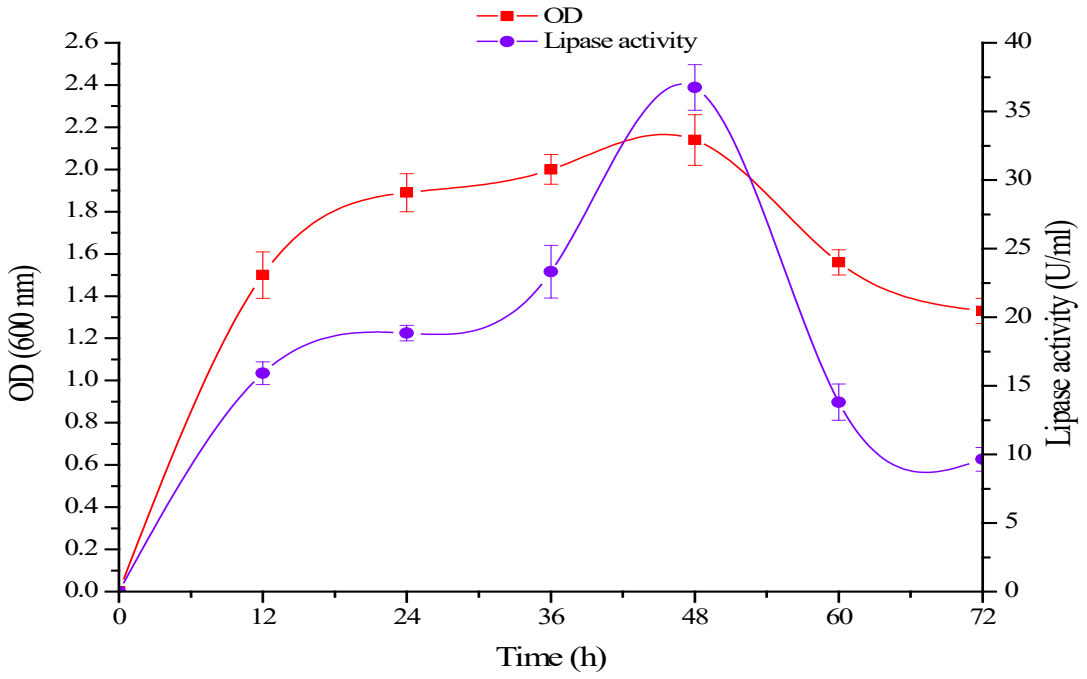

Figure 2. Time course profile of growth and lipase production by *Bacillus aryabhattai* SE3-PB cultivated in a basal medium at 37 °C and 130 rpm during 72 h incubation. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Selection of inducer oil for maximum lipase production

The supplementation of fermentation medium with inducible substrates enhances lipase synthesis and promotes its purification and utilization, since the carbon chain moiety of the fatty acids present in triacylglycerol controls lipase production [50]. In the present study, olive oil in the fermentation media with lipase production of 36.75 U/ml was replaced with commercial vegetable oils (1%, v/v) viz. castor oil, sunflower oil, rice bran oil, canola oil or palm oil in order to determine best oil that will induce maximum extracellular lipase production by *Bacillus aryabhattai* SE3-PB. Lipase production varies in the presence of the different inducer oils, maximum lipase production (72.32 U/ml) recorded in the fermentation media containing sunflower oil as inducer (Figure 3). Lipase production decreased in the order: sunflower oil > canola oil > palm oil > castor oil > rice bran oil. This may be due to variation in fatty acid composition of the vegetable oils, which play a key role in the enzyme production [60]. Maximum lipase production by *Rhizopus delemar* [27], *Staphylococcus chromogenes* 01 A [51], and *Fusarium verticillioides* [56] in the presence of sunflower oil as an inducible substrate has been reported. Sunflower oil was then selected as inducer for further optimization of lipase production by RSM.

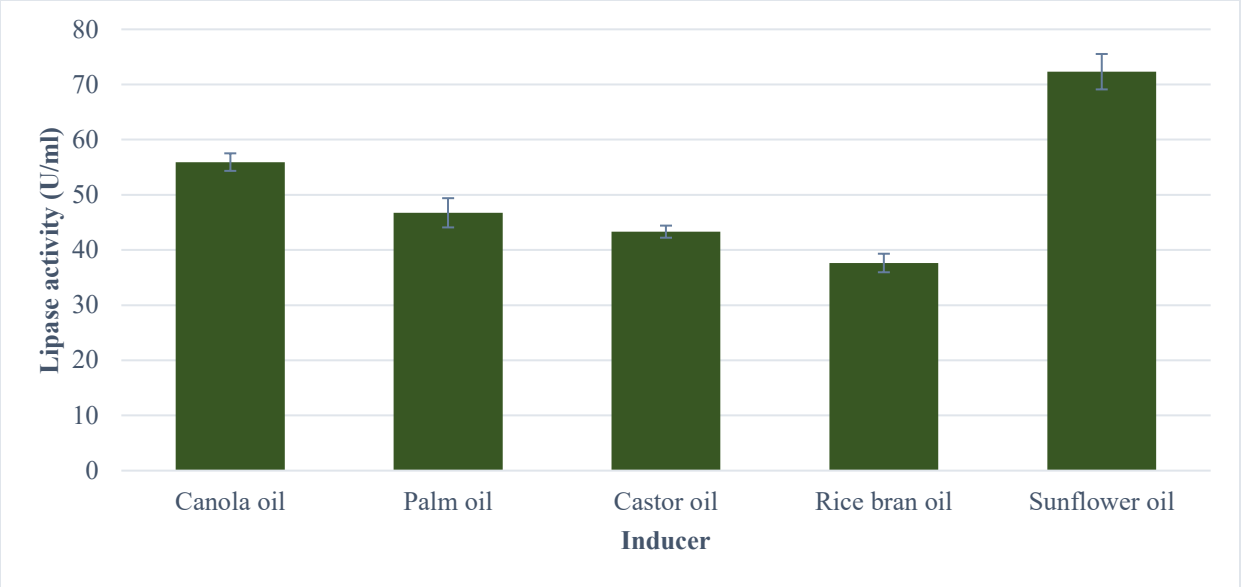

Figure 3. Effect of inducer oils on lipase production by *Bacillus aryabhattai* SE3-PB grown in a basal medium at 37 °C (130 rpm) for 48 h. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Optimization of culture conditions for improved lipase production using response surface methodology

Response surface methodology was used for establishing interactions of five independent parameters including temperature, pH, inoculum volume, agitation and inducer oil concentration, and to determine their optimum conditions for maximum lipase production. A set of 50 experimental runs were generated by the FCCCD; the results of the predicted and experimental responses (lipase production) are presented in Table 2. A response surface quadratic model for lipase production was established along with the independent variables. Lipase production (Y) expressed as a function of temperature (X_1), pH (X_2), inoculum volume (X_3), agitation (X_4) and inducer oil concentration (X_5) is illustrated in the second-order polynomial equation in eq. (2):

$$Y = +119.77 + 21.43X_1 + 13.30X_2 + 11.28X_3 + 9.59X_4 + 10.96X_5 + 2.49X_1*X_2 + 5.45X_1*X_3 + 6.72X_1*X_4 + 12.50X_1*X_5 + 4.45X_2*X_3 + 6.98X_2*X_4 + 6.06X_2*X_5 + 7.36X_3*X_4 + 3.12X_3*X_5 + 10.36X_4*X_5 - 1.84X_1^2 + 1.22X_2^2 + 0.97X_3^2 - 1.58X_4^2 + 0.22X_5^2 \quad (2)$$

The results obtained were then subjected to the analysis of variance (ANOVA) in order to understand the statistical significance of the second-order polynomial equation. The model generated was found to be statistically significant with a value of "Probability > F" less than 0.0500 ($P < 0.0001$), an indication that lipase production could be well described with this model (Table 3). The F-value (178.26) showed that the

model was significant and suitable for simulation of lipase production with any interaction of the five variables [52]. There is only a 0.01% chance that a large "model *F*-value" could occur due to noise. The high *F*-value and very low probability depict the model good prediction of lipase production.

Table 2. FCCCD of five independent variables with predicted and observed lipase production.

Std	Run	X_1: Temperature	X_2: pH	X_3: Inoculum volume	X_4: Agitation	X_5: Inducer oil concentration	Observed response (U/ml)	Predicted response (U/ml)
1	1	30	6	1	100	1	114.8	111.6
28	2	40	8	1	200	3	190.3	186.7
7	3	30	8	3	100	1	105.6	108.9
30	4	40	6	3	200	3	185.3	183.6
12	5	40	8	1	200	1	119.7	115.6
18	6	40	6	1	100	3	117.0	114.2
41	7	35	7	2	150	1	106.9	107.2
31	8	30	8	3	200	3	156.8	153.0
43	9	35	7	2	150	2	124.5	127.6
48	10	35	7	2	150	2	124.5	127.6
2	11	40	6	1	100	1	112.1	108.9
24	12	40	8	3	100	3	171.9	173.7
49	13	35	7	2	150	2	124.5	127.6
37	14	35	7	1	150	2	112.5	109.0
44	15	35	7	2	150	2	124.5	127.6
8	16	40	8	3	100	1	121.6	119.5
5	17	30	6	3	100	1	97.9	98.66
33	18	30	7	2	150	2	93.5	94.99
27	19	30	8	1	200	3	115.8	113.0
36	20	35	8	2	150	2	134.7	131.7
45	21	35	7	2	150	2	124.5	127.6
40	22	35	7	2	200	2	129.8	125.8
3	23	30	8	1	100	1	109.6	107.1
10	24	40	6	1	200	1	92.5	90.09
19	25	30	8	1	100	3	86.4	88.0
26	26	40	6	1	200	3	140.1	137.2
35	27	35	6	2	150	2	110.7	109.4
39	28	35	7	2	100	2	111.8	108.7
25	29	30	6	1	200	3	76.9	73.3
23	30	30	8	3	100	3	98.5	92.62
22	31	40	6	3	100	3	132.1	127.1
16	32	40	8	3	200	1	169.5	164.9
9	33	30	6	1	200	1	79.8	76.22
32	34	40	8	3	200	3	254.7	253.5
20	35	40	8	1	100	3	137.9	133.0
38	36	35	7	3	150	2	132.0	130.6
13	37	30	6	3	200	1	86.5	85.59
34	38	40	7	2	150	2	145.9	139.0
47	39	35	7	2	150	2	124.5	127.6
50	40	35	7	2	150	2	124.5	127.6
21	41	30	6	3	100	3	75.0	72.08
11	42	30	8	1	200	1	88.9	86.35
29	43	30	6	3	200	3	99.3	97.7
46	44	35	7	2	150	2	124.5	127.6
4	45	40	8	1	100	1	104.6	101.9
17	46	30	6	1	100	3	79.1	76.99
6	47	40	6	3	100	1	114.8	112.4
14	48	40	6	3	200	1	125.6	121.1
15	49	30	8	3	200	1	120.1	117.8
42	50	35	7	2	150	3	134.9	130.9

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model for optimization of lipase production.

Source	SS	df	MS	*F*-value	*P* value
Model	49633.85	20	2481.69	178.26	< 0.0001[a]
X_1	15608.76	1	15608.76	1121.20	< 0.0001[a]
X_2	6012.66	1	6012.66	431.90	< 0.0001[a]
X_3	4327.91	1	4327.91	310.88	< 0.0001[a]
X_4	3127.68	1	3127.68	224.67	< 0.0001[a]
X_5	4087.20	1	4087.20	293.59	< 0.0001[a]
X_1*X_2	197.91	1	197.91	14.22	0.0007[a]
X_1*X_3	950.70	1	950.70	68.29	< 0.0001[a]
X_1*X_4	1444.26	1	1444.26	103.74	< 0.0001[a]
X_1*X_5	5003.50	1	5003.50	359.41	< 0.0001a
X_2*X_3	634.93	1	634.93	45.61	< 0.0001[a]
X_2*X_4	1557.66	1	1557.66	111.89	< 0.0001[a]
X_2*X_5	1174.43	1	1174.43	84.36	< 0.0001[a]
X_3*X_4	1732.54	1	1732.54	124.45	< 0.0001[a]
X_3*X_5	311.38	1	311.38	22.37	< 0.0001[a]
X_4*X_5	3436.62	1	3436.62	246.86	< 0.0001[a]
Residual	403.72	29			
Lack of fit	241.91	22	11.00	0.48	0.9134
Pure error	161.81	7	23.12		
Cor. Total	50037.58	49			

SS: Sum of squares; df: degree of freedom; MS: Mean square; *F*: *F* ratio; *P*: probability value;

R^2 = 0.9919; Adj. R^2 = 0.9864; Pred. R^2 = 0.9781; Adeq. precision = 74.857; [a], statistically significant ($P < 0.05$)

The coefficient of determination (R^2) is an index of variance that lies between 0 and 1, with 1 suggesting the effectiveness of the model to navigate the design accurately and 0 showing a total inability [57]. The closer the value of R^2 to 1, the stronger the model and better its prediction efficiency of the response [58]. The R^2 value of 0.9919 signified a relatively high similarity between experimental and predicted values. This also implied that, 99.19% variability of the response could be explained by the model and only about 0.81% of the entire variation cannot be explained by the model [53,54]. The predicted R^2 value of 0.9781 was found to be in accordance with the adjusted R^2 value of 0.9864. This suggested that the regression model could be used to describe the response trends [15,30].

A satisfactory correlation between experimental and predictive values is shown by the predicted versus actual plot (Figure 4). The clustered points around the diagonal indicates a good fit of the model.

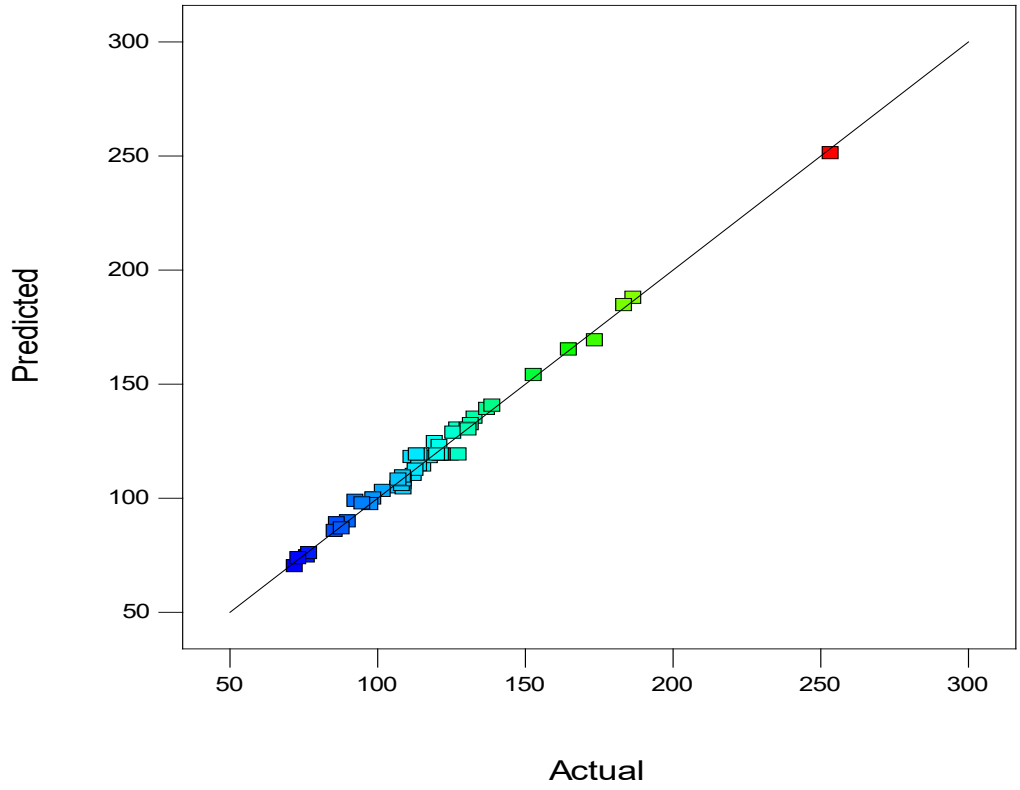

Figure 4. Predicted versus actual response plot for lipase production by *Bacillus aryabhattai* SE3-PB.

The interaction effects and optimum conditions of the independent variables optimized for enhanced lipase production are presented by contour plots showed in Figure 5A-J. Two variables were analyzed at a time while keeping other variables at fixed levels (center point) using a Design Expert 10 software. Significant interaction was observed among the variables, as illustrated by elliptical contour plot obtained in the case of inducer oil concentration and agitation.

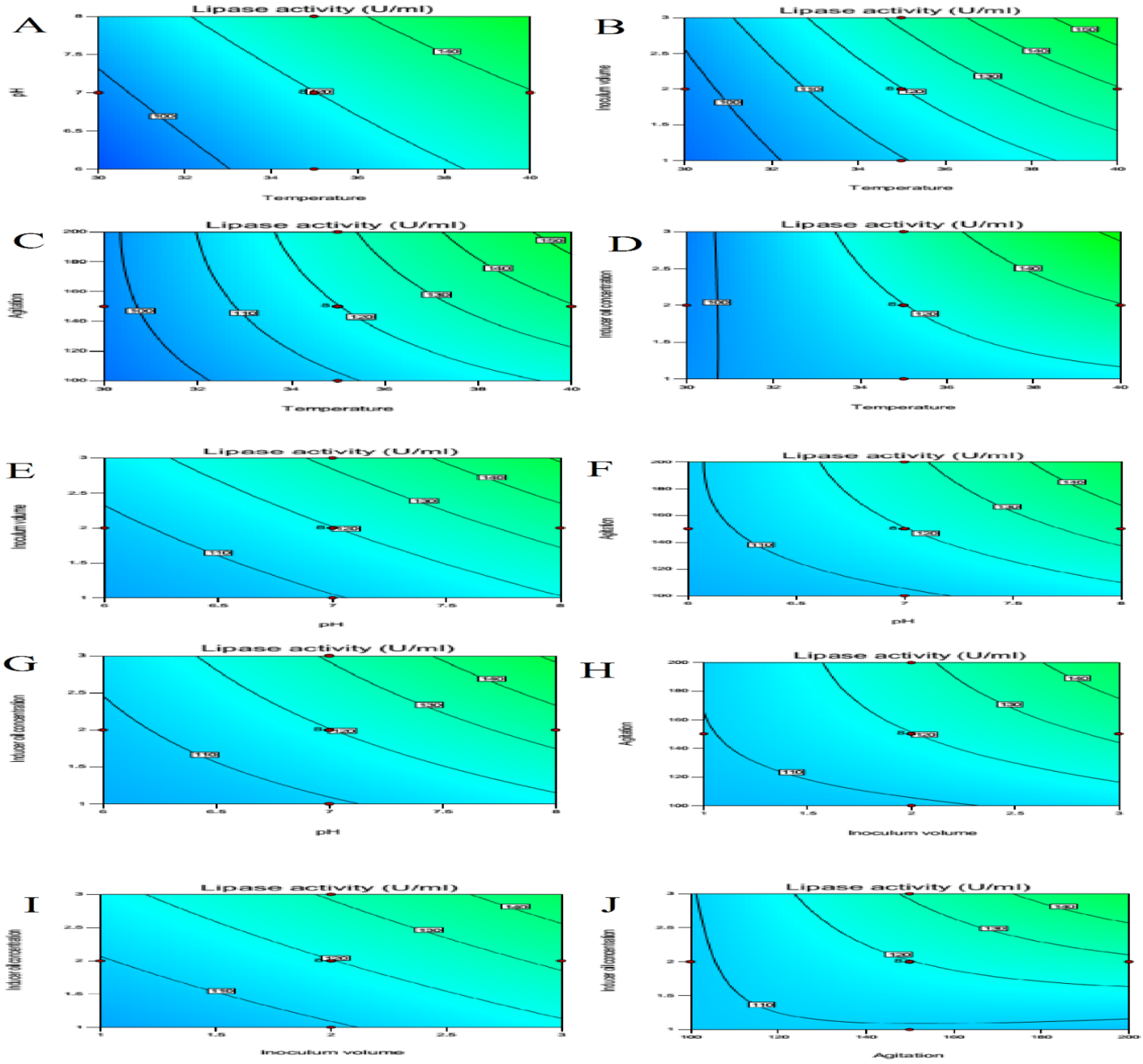

Figure 5. Contour plots of lipase production by *Bacillus aryabhattai* SE3-PB showing interaction between (A) temperature and pH, (B) inoculum volume and temperature, (C) temperature and agitation, (D) inducer oil concentration and temperature, (E) inoculum volume and pH, (F) agitation and pH, (G) inducer oil concentration and pH, (H) agitation and inoculum volume, (I) inducer oil concentration and inoculum volume, and (J) inducer oil concentration and agitation.

Experimental validation of the optimized process conditions

The predicted optimization result by the model suggested that maximum lipase production (265.82 U/ml) by *Bacillus aryabhattai* SE3-PB could be achieved when temperature, pH, inoculum volume, agitation and inducer oil concentration were set at 40 °C, 7.6, 2.8% (v/v), 193 rpm and 2% (v/v), respectively. This was further confirmed by carrying out a laboratory scale experiment in shake flasks under the conditions predicted by the model. Results obtained showed a maximum mean lipase production of 264.02 ± 1.94 U/ml (60 h) (Figure 6) during late log phase, found to be in agreement with the predicted value. This implied an approximately 98.9% of validity attained, an indication that the model gave an adequate prediction on lipase production. Optimization of culture conditions by *Bacillus aryabhattai* SE3-PB led to an approximately 7.2-fold increase in lipase production when using the unoptimized medium as reference. A high correlation observed between the predicted and experimental results confirmed the accuracy and applicability of the model for process optimization studies. The statistical experimental design and analysis were found to be efficient in elucidating the significant parameters and respective optimum conditions for maximum lipase production. Improvement of lipase production by *Bacillus aryabhattai* SE3-PB using FCCCD was considerably higher than that of *Burkholderia* sp. [15,55], *Enterobacter aerogenes* [26], and *Acinetobacte*r sp. AU07 [28].

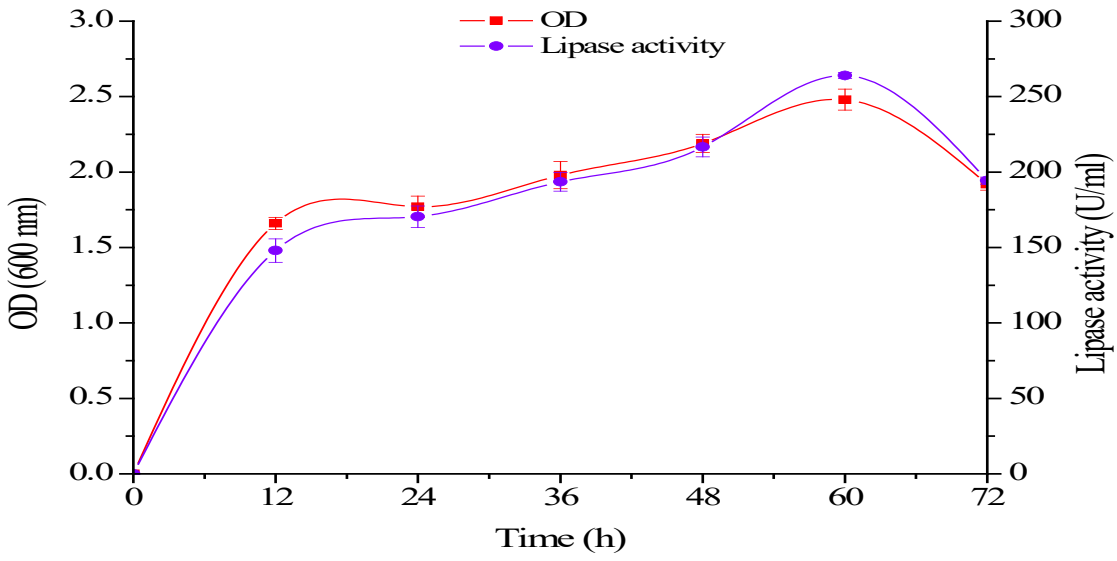

Figure 6. Time course profile of growth and lipase production by *Bacillus aryabhattai* SE3-PB under the predicted optimal conditions. The culture medium consisted of 2.8% (v/v) inoculum volume and 2% (v/v) inducer oil concentration with initial pH adjusted to 7.6, and incubated at 40 °C at 193 rpm. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Conclusions

In the present study, optimization of culture conditions for enhanced extracellular lipase production by *Bacillus aryabhattai* SE3-PB was investigated using FCCCD. Lipase production was found to be significantly influenced by temperature, pH, inoculum volume, agitation and inducer oil concentration. The high similarity of observed lipase production with the predicted lipase yield confirmed the validity and accuracy of the generated model. An overall 7.2-fold increase in lipase production was recorded after optimization of culture conditions by RSM. This study further reiterates the use of FCCCD in determining significant parameters and optimum conditions for maximum production of desired microbial metabolite. Optimum conditions of the parameters investigated will provide a baseline for further study involving large scale and cost-effective production of lipase by *Bacillus aryabhattai* SE3-PB.

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Disclosure statement

The authors declare that there are no conflicts of interest.

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CHAPTER EIGHT

PARTIAL PURIFICATION, IMMOBILIZATION AND CHARACTERIZATION OF ALKALINE PROTEASE FROM AN INDIGENOUS *BACILLUS ARYABHATTAI* AB15-ES ISOLATED FROM LIPID-RICH WASTEWATER

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Partial purification, immobilization and characterization of alkaline protease from an indigenous *Bacillus aryabhattai* Ab15-ES isolated from lipid-rich wastewater

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Abstract In the present study, partially purified alkaline protease from *Bacillus aryabhattai* Ab15-ES was immobilized in alginate gel beads by entrapment method. Maximum activities of 68.76 and 71.06% were recorded at optimum conditions of 2% (w/v) sodium alginate and 0.3 M calcium chloride, respectively for the entrapped enzyme. Biochemical and kinetic properties of free and immobilized protease were investigated by determining their activity and stability profiles in terms of pH and temperature. Both enzyme preparations exhibited maximum activity at optimum pH and temperature of 8.0 and 50 °C. However, in comparison to free enzyme, the immobilized protease showed improved stability at pH 8.0-9.0 and at temperature range of 40-50 °C. In addition, the immobilized protease exhibited higher V_{max} and increased (1.65-fold) substrate affinity than soluble enzyme. The immobilized protease was found to be more stable than free enzyme, retaining 80.88 and 38.37% of its initial activity when stored at 4 and 30 °C, respectively. After repeated use for seven times, protease entrapped in alginate beads maintained 32.93% of its original activity. These findings suggest enhanced catalytic efficiency and sustainable use of the developed immobilized protease for various industrial and environmental applications.

Keywords *Bacillus aryabhattai* Ab15-ES; Alkaline protease; Immobilization; Alginate beads; Enzyme kinetics

Introduction

Proteases are enzymes that catalyze the hydrolysis of proteins into simpler subunits by cleavage of peptide bond that exists between two amino acids of a polypeptide chain [1-3]. They occupy a pivotal position, constituting more than 65% of total industrial enzyme market [4-6], which is projected to increase further in coming years [7]. Protease have versatile applications in various industries including detergent, pharmaceutical, leather, silk, food, silver recovery and agriculture as well as in wastewater treatment and

organic chemical synthesis [6,8-10]. Proteases are ubiquitous in nature, and are produced from plants, animals and microbes [11]. The inability of plant and animal proteases to meet contemporary global demand has led to an increased interest in microbial proteases. Microbial proteases serve as an excellent source of commercial enzyme since microorganisms can be cultivated in large quantities at relatively short time through an established fermentation process for the generation of abundant yield of the desired metabolite. They also have broad biochemical diversity and are susceptible to genetic manipulation [12]. Microbial proteases account for approximately 40% of the total worldwide production of the enzyme [13-15]. Microorganisms such as bacteria, molds and yeasts have been reported for extracellular protease production [16-17].

Free enzymes are generally soluble, unstable and susceptible to harsh reaction conditions including extreme temperature, very high or low pH, high ionic strength, high concentration of reactants and presence of inhibitors [18]. Enzymes may not function optimally under such extreme conditions which are often encountered in bioprocesses [19]. In order to ameliorate the aforementioned challenges, immobilization of protease in suitable support materials represents a promising alternative [7,20-22]. The use of immobilized enzymes in bioprocesses has many advantages including increased stability and activity over broad range of pH and temperatures, localization, ease of product separation and handling, recovery yield and reusability, rapid termination of reactions, controlled product formation, adaptability to various engineering designs with a consequent decrease in running cost and autolysis rate [18,23]. However, selection of suitable support materials and immobilization techniques is paramount to overcome the drawbacks of immobilization. The general methods employed for enzyme immobilization include physical adsorption, ionic binding, covalent binding, cross-linking and entrapment methods [24]. Amongst the different immobilization techniques, entrapment method has been mostly considered due to its simplicity and non-toxicity [25].

Entrapment method involves physical restriction of the enzyme within a confine space or network in a semi-permeable capsule, obtained by gelation of polyanionic or polycationic polymers in the presence of multivalent counterions thus, retaining enzyme catalytic activity and capacity to be used continuously [7,26,27]. It is carried out in a 3-dimensional gel lattice, made of either natural (agar, cellulose, alginate, carrageenan) or synthetic (polyacrylamide, polyurethane, polyvinyl, polypropylene) polymers [28,29]. However, calcium alginate beads are mostly preferred owing to their mild gelling properties, easy formulation, biocompatibility, cost-effectiveness and potential to improve enzyme stability and functional properties [30-32]. Alginate is a water-soluble anionic linear polysaccharide composed of sequential arrangements of 1,4-linked β-D-mannuronic acid and α-L-glucuronic acid in different proportion, and can be precipitated by addition of Ca^{2+} ions, producing microspheres with good strength and flexibility [33-34].

It is employed in many biotechnological applications as support material for releasing and encapsulating cells and enzymes [35].

Proteases are classified as acidic proteases, neutral proteases and alkaline proteases based on optimal pH of activity [11,36]. Alkaline proteases are proteases that are active at neutral to alkaline pH conditions. They occur as serine or metalloproteases, and are commonly exploited for various commercial applications due to their higher activity and stability at broad pH and temperature ranges [37-39]. Many microbial species are known as alkaline protease producers. However, among bacteria, members of the genus *Bacillus* including *Bacillus pumilus* [40], *Bacillus licheniformis* [41], *Bacillus caseinilyticus* [42], *Bacillus subtilis* [43-44], *Bacillus megaterium* [45], *Bacillus coagulans* [46] and *Bacillus alveayuensis* [6] are notable for commercial scale production of alkaline proteases for various biotechnological and environmental applications [8,47].

Due to the emergent market and potential application of proteases, there is a continuing interest in the isolation of indigenous hyperactive proteolytic bacteria with robust catalytic activity suitable for various industrial and environmental applications. In the present study, extracellular alkaline protease from *Bacillus aryabhattai* Ab15-ES isolated from poultry processing wastewater was partially purified and immobilized by entrapment in alginate beads. Thereafter, comparative study of biochemical and kinetics properties of the free and immobilized proteases was carried out to ascertain their possible biotechnological applications.

Materials and Methods

Partial purification of protease

Bacillus aryabhattai Ab15-ES was cultivated in optimized medium consisting in g/L: Maltose, 12.35; Beef extract, 5.30; NaCl, 0.5; $CaCl_2.2H_2O$, 0.1; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgSO_4.7H_2O$, 0.1; pH was adjusted to 7.8. The inoculated culture medium was then incubated at 40 °C for 48 h at 150 rpm. The cell free culture supernatant (crude protease) obtained by centrifugation at 10000 rpm for 10 min at 4 °C was partially purified by ammonium sulphate precipitation and dialysis. Solid ammonium sulphate was added to the crude enzyme by continuous stirring up to 60% saturation. The saturated solution was kept undisturbed at 4 ºC overnight. The precipitate was collected by centrifugation at 10000 rpm for 10 min at 4 ºC, re-dissolved in a small amount (1:50) of 0.05 M Tris-HCl buffer (pH 7.2) and then dialyzed overnight against the same buffer at 4 °C.

Immobilization of partially purified protease in calcium alginate gel beads

Immobilization of protease was carried out according to the entrapment method of Guleria *et al*. [7] with modifications using sodium alginate as support material. Varying amounts of sodium alginate were added to 0.05 M Tris-HCl buffer (pH 7.2) to attain different concentrations (1-5%). Two milliliter aliquot of sodium alginate suspension was mixed with 0.5 ml of the partially purified protease. This mixture was extruded dropwise into a 5 ml $CaCl_2$ solution (0.1- 0.5 M) using a hypodermic syringe from a 5 cm height with constant stirring. The beads obtained were kept at 4 °C for 1 h for curing. Thereafter, the cured beads of about 3 mm diameter were recovered from the solution by filtration, washed 3-4 times under mild agitation with 0.05 M Tris-HCl buffer (pH 7.2) and distilled water to remove unbound enzyme, and then stored at 4 °C until use [26]. A similar method was used for the preparation of control alginate beads without the enzyme.

Protease assay

Protease activity was determined according to the modified method of Secades and Guijarro [48] using azocasein as a substrate. Enzyme solution (120 µl) was added to 480 µl of azocasein solution (1%, w/v of azocasein in 0.05 M Tris-HCl buffer, pH 7.2), and the mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 600 µl of 10% chilled trichloroacetic acid (TCA) followed by further incubation on ice for 30 min to allow undigested protein to precipitate. Thereafter, coagulated protein was removed from the reaction mixture by centrifugation at 10000 rpm for 10 min at 4 °C using high- speed refrigerated centrifuge (Avanti J-26 XP, Beckman Coulter, USA). The obtained supernatant (800 µl) was neutralized by adding 200 µl of 1.8 N NaOH. The absorbance was measured at 440 nm using a UV-Vis spectrophotometer (UVmini-1240, Schimadzu, Australia). The blank consisted of TCA-substrate solution. The activity of immobilized protease was determined using the procedure described above, except that 0.5 g of microspheres was used for the assay. Lowry method was used for the estimation of total protein using bovine serum albumin as a standard [49]. One unit (U) of enzyme activity was defined as the amount of enzyme that yielded an increase in absorbance (440 nm) of 0.01 per minute under defined assay conditions.

Determination of immobilization efficiency

The immobilization efficiency, *Y* described as the percentage of bound enzyme activity observed in the immobilizate is calculated using equation (1) [50]:

$$Y\ (\%) = \frac{Observed\ activity}{Immobilized\ activity} X\ 100 \qquad (1)$$

Where immobilized activity was determined by measuring the total residual enzyme activity that remains in the enzyme solution after immobilization and subtracting this from the total starting activity [50].

Characterization of free and immobilized protease

Effect of temperature and pH on the activity of free and immobilized protease

The temperature at which optimum protease activity was achieved for both free and immobilized proteases was determined by carrying out enzyme assay at various temperatures (30-80 ºC) for 30 min in 0.05 M Tris-HCl buffer (pH 7.2). Protease activity was measured, as described previously. The optimum pH for free and immobilized protease activity was determined at the optimum temperature for 30 min at different pH values ranging from 3.0-11.0. The buffers used include: 0.05 M citrate buffer (pH 3.0-4.5), 0.05 M acetate buffer (pH 5.0-5.5), 0.05 M phosphate buffer (pH 6.0-7.0), 0.05 M Tris-HCl buffer (pH 7.5-9.0), and Glycine-NaOH buffer (9.5-11.0). Azocasein was dissolved in the respective buffers, and then used to assay for protease activity, as described above. The relative protease activities as percentage (%) of maximum enzyme activity were calculated and plotted against the respective temperature or pH. All experiments were carried out in triplicate.

Effect of temperature and pH on the stability of free and immobilized protease

The thermal stability of the free and immobilized protease was determined by pre-incubating the enzyme in 0.05 M Tris-HCl buffer (pH 8.0) at different temperatures (40, 45, 50 ºC) for up to 3 h. Aliquots were withdrawn at desired intervals and assayed for residual activity under standard assay conditions. Un-heated enzyme was considered as a control (100%). The pH stability of the enzyme was assessed by pre-incubating the enzyme in the respective buffers (0.05 M) for up to 3 h at the optimum temperature. Aliquots were withdrawn at time intervals, and assayed for residual protease activity under standard experimental conditions. All experiments were done in triplicate.

Kinetics properties of free and immobilized protease

The influence of substrate (azocasein) concentrations ranging from 1 to 10 mg/ml on free and immobilized protease activity was studied by measuring enzyme activity, as previously described under standard assay conditions. Lineweaver-Burk plot was constructed by plotting reciprocals of substrate concentrations against the reciprocals of enzyme velocity. From the plot, Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were determined [51].

Storage stability of free and immobilized protease

The storage stability of free and immobilized enzyme was determined by preserving the enzymes at 4 and 25 ℃. At interval of 5 d for up to 30 d, samples of the stored enzyme were assayed for protease activity. Residual activity was calculated against the initial enzyme activity taken as 100%.

Reusability potential of the immobilized protease

In order to assess the reusability potential of the protease entrapped in the calcium alginate beads, the azocasein hydrolysis reaction was repeated several times at 37 °C for 30 min. Thereafter, after every use, the beads were separated, washed with Trs-HCl buffer (pH 7.2) and added into fresh substrate solution. Protease activity was measured after every reaction cycle and relative activity was calculated by comparing the enzyme activity to that of the freshly prepared beads in the first cycle taken as 100%.

Scanning electron microscopy of beads with immobilized protease

The surface morphology of alginate beads containing immobilized protease was studied using ZEISS EVO LS15 scanning electron microscope.

Results and Discussion

Partial purification of protease

Bacillus aryabhattai Ab15-ES was cultivated in a previously optimized fermentation medium for extracellular protease production. The results of the purification procedures of obtained crude enzyme are summarized in Table 1. A 2.19-fold increase in specific activity was obtained for the partially purified protease compared to the crude enzyme, with a recovery of 60.98%. Sahin et al. [22] and Zhu et al. [52] reported a 2.1-fold purification and 64% recovery, respectively following ammonium sulphate precipitation of protease from *Bacillus subtilis* M-11 and *Geobacillus* sp. YMTC 1049. Similarly, Mothe et al. [42] recorded a 1.65-fold increase in protein purification with 48% recovery during ammonium sulphate precipitation of protease from *Bacillus caseinilyticus*.

Table 1 Summary of purification steps of protease from *Bacillus aryabhattai* Ab15-ES

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme	24784	190.31	130.23	100	1.00
$(NH_4)_2SO_4$ precipitation (60%)	15114	52.90	285.71	60.98	2.19

Protease immobilization and scanning electron microscopic analysis

Enzyme entrapment depends on sodium alginate and calcium chloride concentrations for the formation of beads with high permeability and rigidity [53]. In this study, the effects of sodium alginate concentrations ranging from 1-5% (w/v) and 0.2 M $CaCl_2$ was investigated on the immobilization efficiency of protease from *Bacillus aryabhattai* Ab15-ES. Maximum immobilization efficiency (68.76 ± 2.42%) was recorded with beads prepared from 2% sodium alginate (Table 2). After which, there was a gradual decrease in the immobilization efficiency with lowest immobilization (22.78 ± 2.07%) obtained with 1% sodium alginate. This may probably be as a result of maximum outflow of the enzyme from the beads, resulting from larger pore sizes of the beads and less tight cross-links with calcium chloride, leading to lower immobilization efficiency [21,53]. This corroborates the findings of Geethanjali and Subash [53] and Anwar et al. [20] in which maximum immobilization efficiency was recorded from 2% sodium alginate. In addition, sodium alginate concentrations of 2-3% have been reported by several authors to be suitable for enzyme immobilization [7,21,54-56].

Table 2 Immobilization efficiency of alkaline protease from *Bacillus aryabhattai* Ab15-ES at varying sodium alginate concentrations

Sodium alginate concentration (%)	Immobilization efficiency (%)
1	22.78 ± 2.07
2	68.76 ± 2.42
3	53.90 ± 3.86
4	45.35 ± 1.62
5	39.74 ± 0.88

*All values are expressed as mean from triplicate values; ± indicates SD

Since calcium chloride serves as a cross-linking agent, its concentration affects the activity and density of immobilized biomolecules [57]. This also influences the mechanical strength of alginate beads as well as efficiency of immobilized systems [58]. Therefore, the concentration of calcium chloride is essential for the stability and pore size of the bead [59]. The influence of calcium chloride concentrations (0.1-0.5 M) on the immobilization efficiency was studied at 2% sodium alginate, since 2% showed the highest immobilization efficiency. Highest entrapped enzyme activity was found from beads prepared from 0.3 M calcium chloride with immobilization efficiency of 71.06 ± 1.30% (Table 3). This supports the findings of Geethanjali and Subash [53] in which maximum immobilization efficiency was recorded with beads formed from 0.3 M calcium chloride.

Table 3 Immobilization efficiency of alkaline protease from *Bacillus aryabhattai* Ab15-ES at varying calcium chloride concentrations

$CaCl_2$ concentration (M)	Immobilization efficiency (%)
0.1	46.81 ± 2.41
0.2	62.96 ± 0.58
0.3	71.06 ± 1.30
0.4	55.92 ± 2.40
0.5	42.87 ± 1.78

*All values are expressed in mean; ± indicates SD

Scanning electron microscopic analysis of the alginate gel beads revealed the presence of irregular pores of various dimensions on the bead surface coupled with a stable microstructure with evenly distributed layers in the epicentre of the beads (Fig. 1). The porosity of the beads helps to enhance its specific surface area and increase substrate diffusion [79].

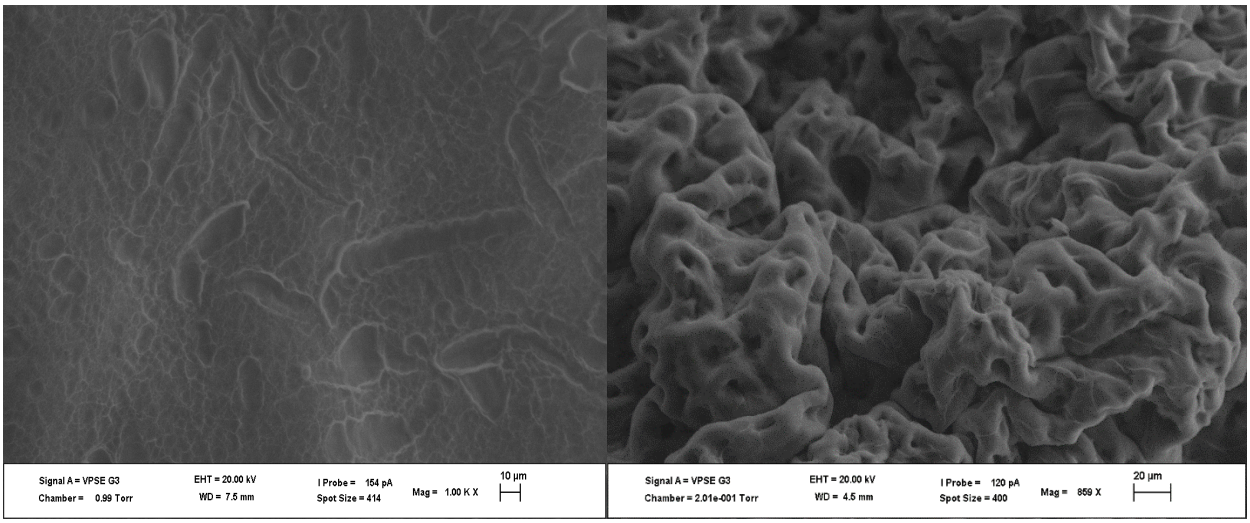

Fig. 1: Scanning electron microscopy of surface morphology of alginate beads.

Effect of temperature and pH on the activity of free and immobilized protease from *Bacillus aryabhattai* Ab15-ES

The optimum temperature of free and immobilized protease activity was determined by carrying out enzyme assay at different temperatures ranging from 30 to 80 °C. An increase in the relative activities of both soluble and immobilized enzyme was observed as the temperature increases from 30 to 50 °C, with maximum activity recorded at optimum temperature of 50 °C (Fig. 2). Thereafter, the relative activities of both free and immobilized protease decreased up to 80 °C. However, the entrapped protease demonstrated higher relative activity when compared to free enzyme, especially at high temperature range (55-80 °C), an indication that the support material played a protective role in retaining the tertiary structure of the enzyme at higher temperatures [20,78]. Similar results have been reported for protease immobilized on polysulfone membrane [22] and Fenugreek β-amylase covalently attached onto functionalized graphene sheets [60]. At 80 °C, the relative activity of the immobilized protease is 1.6-fold higher than that of the soluble enzyme. This clearly indicated that the support material retained the tertiary structure of the enzyme at high temperature [20]. In other words, multi-interactions between the matrix and enzyme play a crucial role in the tolerance to temperature of the immobilized enzyme [61].

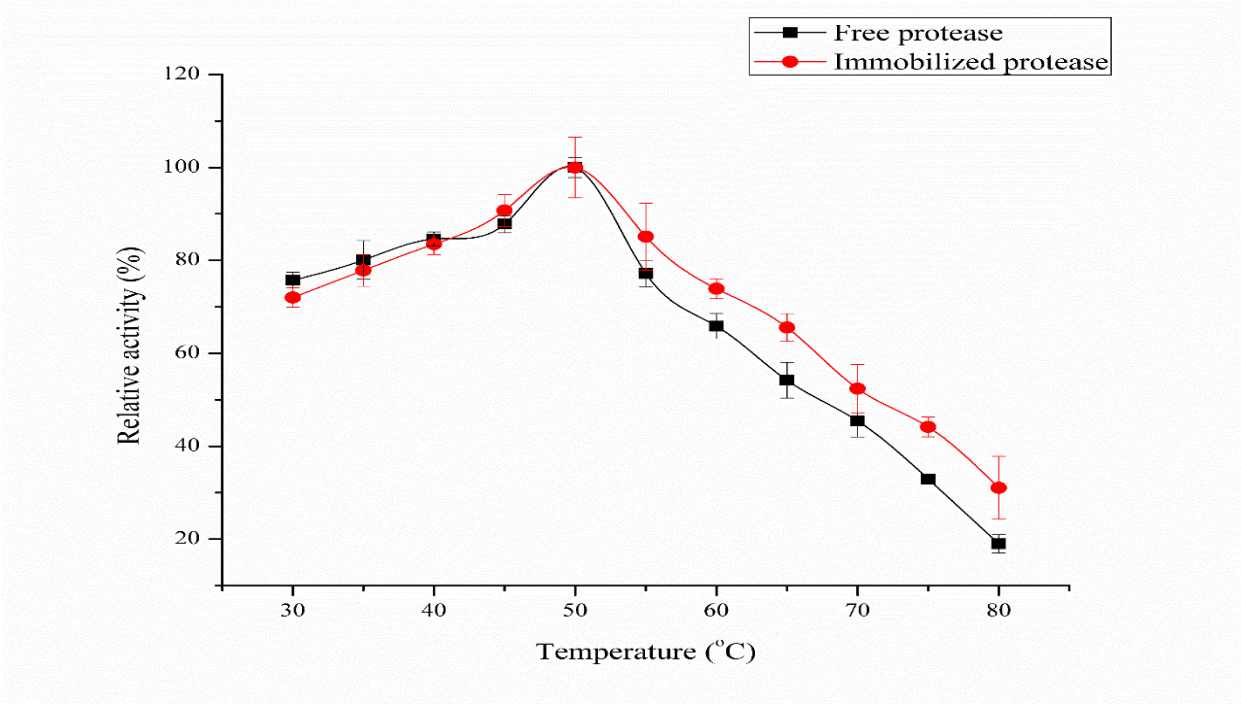

Fig. 2 Effect of temperature on activity of free and immobilized protease from *Bacillus aryabhattai* Ab15-ES. Protease activity was measured under standard assay conditions at various temperatures (30-80 °C) at pH 7.2. Values indicate the average of triplicate values while the error bars represent the standard deviation.

The effect of pH on activity of soluble and entrapped protease was investigated under varied pH values (3.0-11.0) in different buffers at 50 °C, and the results are depicted in Fig. 3. The catalytic activity of the enzyme increases as pH increased, reaching maximum activity at pH 8.0 for both soluble and entrapped enzyme. This suggested that the chemical coupling of the enzyme to the polymer results in no change in its pH profile. Anwar et al. [20] and Geethanjali and Subash [53] have reported similar results on immobilization of proteases from *Bacillus subtilis* KIBGE-HAS and *Labeo rohita*, respectively where no change in the pH profile of the enzyme was observed before and after entrapment in calcium alginate. This phenomenon has also been observed by other authors [62-64]. In addition, the higher relative activity of the immobilized protease compared to the free enzyme within the evaluated pH range (8.5-11.0) suggests its resistance to the alkaline changes in medium [65]. Similar results have also been reported for enzymes immobilized onto/in solid matrix [55,64,66].

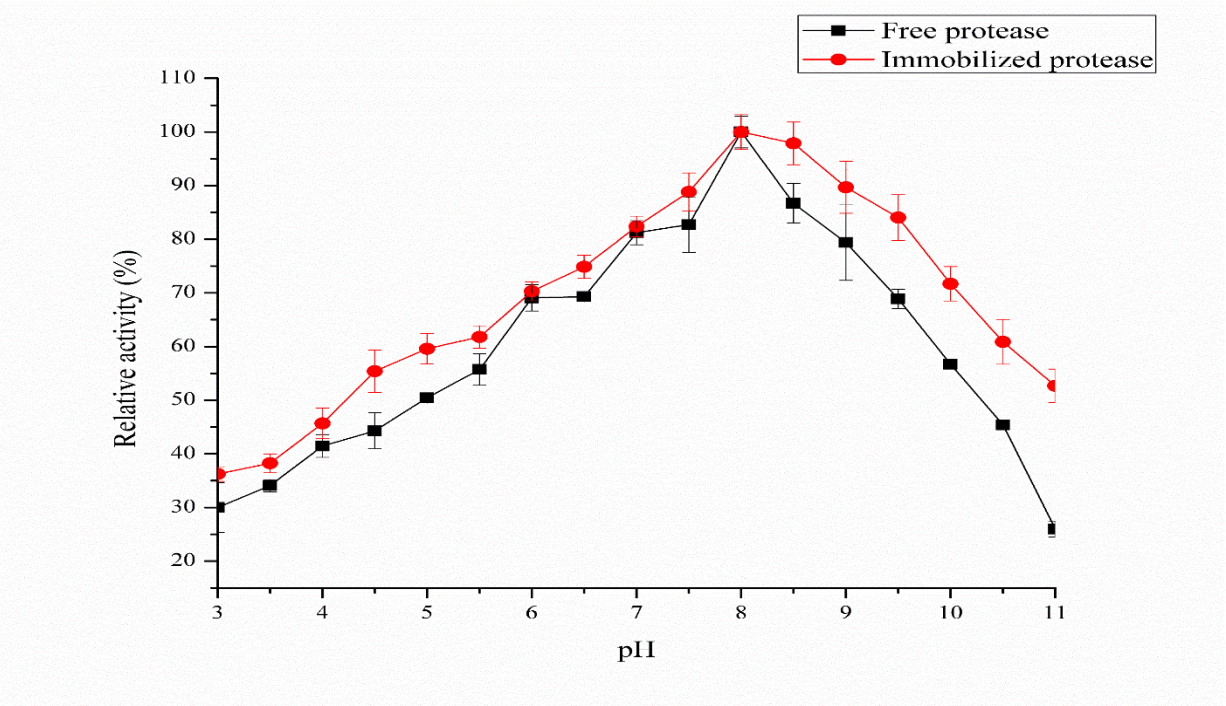

Fig. 3 Effect of pH on activity of free and immobilized protease from *Bacillus aryabhattai* Ab15-ES. Enzyme activity was determined at 50 °C. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Thermal and pH stability of the free and immobilized protease from *Bacillus aryabhattai* Ab15-ES

The thermal stability of free and immobilized protease was investigated in the temperature range of 40-50 °C for 0-180 min. As shown in Fig. 4, the protease immobilized in alginate beads demonstrated better thermal stability compared to free enzyme. After incubation at 50 °C for 3 h, the immobilized enzyme retained 60.58% of its initial activity whereas, the free soluble enzyme retained only 28.41%. The significant improvement in thermal stability of the entrapped protease may be due to immobilization in the support material which protect the tertiary structure of the enzyme and prevent conformational transition of the enzyme upon heating [67,68]. Enhancement of enzyme stability after immobilization in alginate beads has been reported by several authors [55,69-71].

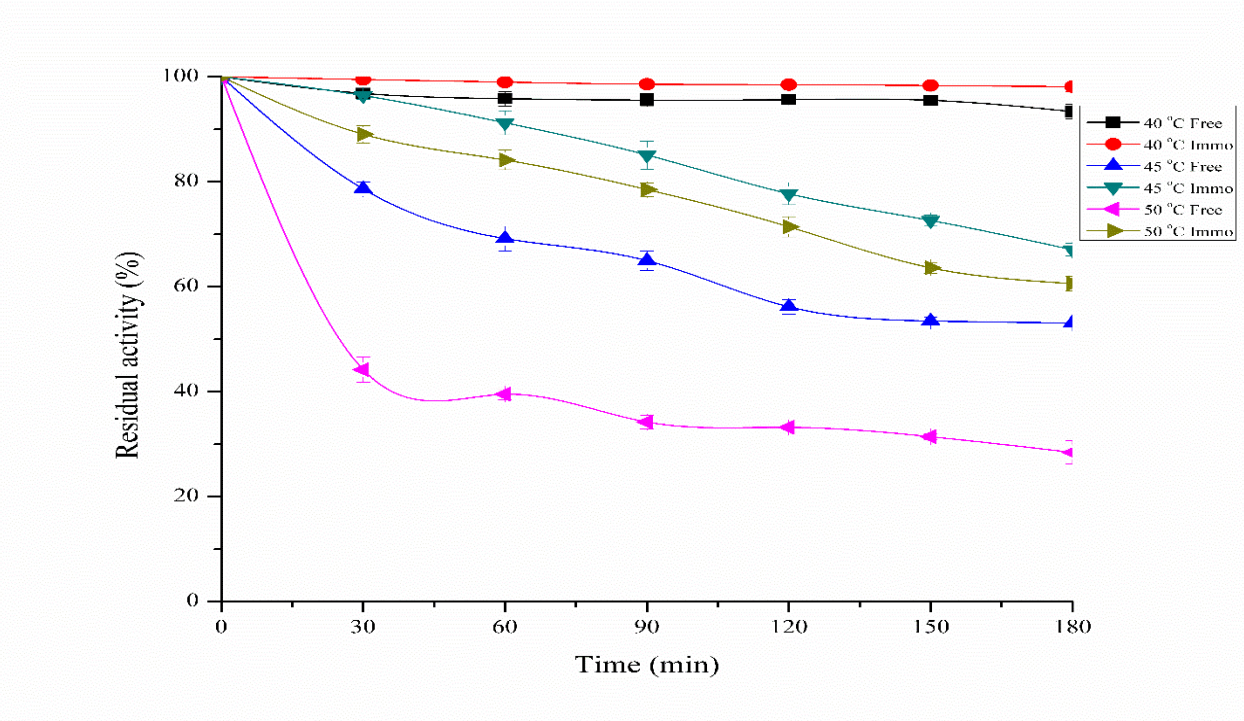

Fig. 4 Thermal stability of free and immobilized protease from *Bacillus aryabhattai* Ab15-ES. The enzyme was pre-incubated at different temperatures (40-50 °C) for 3 h at pH 8.0, and the residual enzyme activities were estimated at regular intervals under standard assay conditions. The non-heated enzymes were taken as 100%. Values indicate the average of triplicate values while the error bars represent the standard deviation.

The pH stability was investigated by pre-incubating the free or immobilized protease in different buffers (pH 8.0-9.0) for 3 h at optimum temperature. Samples were taken at 30 min intervals for determination of residual enzyme activity. The entrapped enzyme in alginate beads demonstrated higher stability than free enzyme, especially at pH 8.5 and 9.0, with retention of 69.6 and 63.53% activity, respectively after 3 h (Fig. 5). These findings can be attributed to the enzyme protection provided by the microenvironment structure and properties of the alginate beads. Similar results have been reported during immobilization of thermostable β-glucosidase in alginate gel beads and κ-carrageenase onto magnetic iron oxide nanoparticles [55,64].

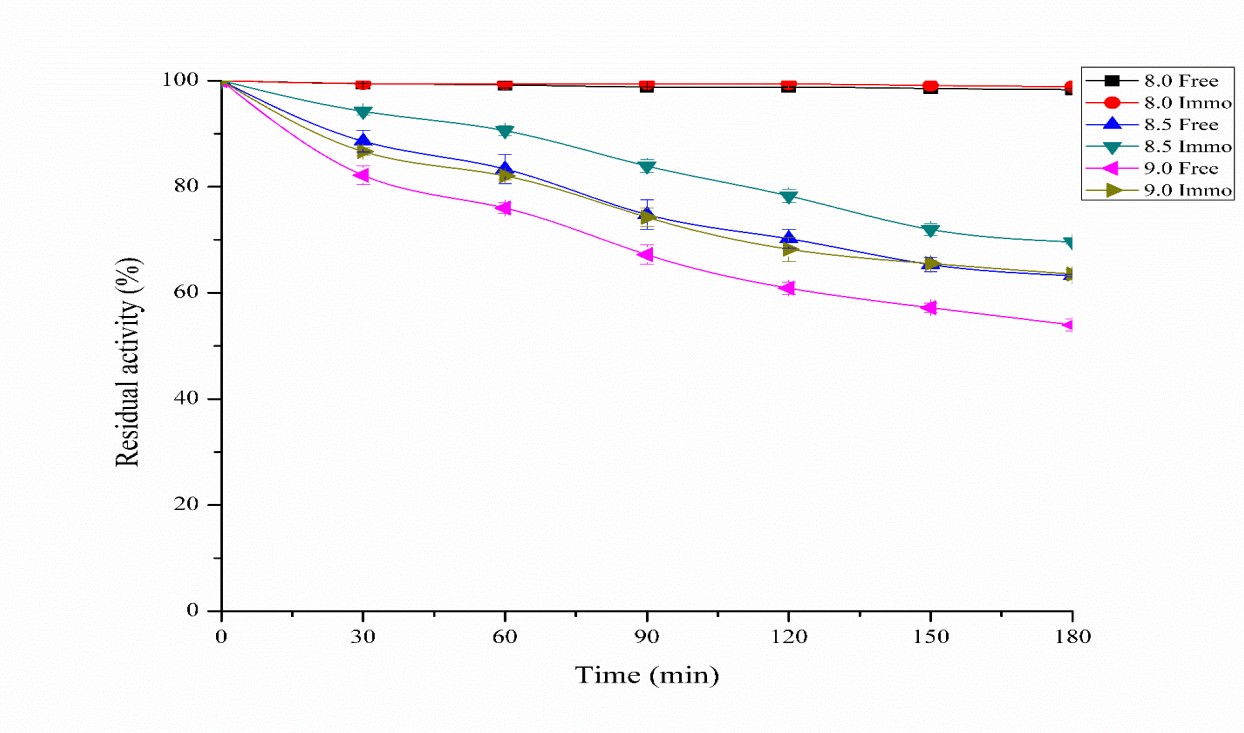

Fig. 5 pH stability of free and immobilized protease from *Bacillus aryabhattai* Ab15-ES. The enzyme was pre-incubated at pH values (8.0-9.0) for 3 h at 50 °C, and the residual enzyme activities were estimated at regular intervals under standard assay conditions. The non-heated enzymes were taken as 100%. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Kinetics properties of free and immobilized protease

The kinetics properties represent a vital factor for determining the efficiency of the immobilization process. In the present study, kinetics parameters (K_m and V_{max}) were estimated from Lineweaver-Burk plot using azocasein as a substrate at pH 8.0 and 50 °C. The calculated K_m values of free and immobilized protease were 2.023 and 1.225 mg/ml, respectively. The lower K_m value of the immoblized enzyme indicates increased affinity (1.65-fold) of the biocatalyst toward the substrate following entrapment in alginate gel beads (Fig. 6). Furthermore, the V_{max} of the immobilized protease was higher (250 U/ml) than that of the free enzyme (232.56 U/ml), suggesting a more efficient conformation of the immobilized enzyme in the solid matrix [72]. In addition, the V_{max}/K_m value of the immobilized enzyme was found to be higher than the corresponding value of the free counterpart (204.08 U/mg *vs* 114.96 U/mg), an indication of high catalytic efficiency of the immobilized enzyme [78].

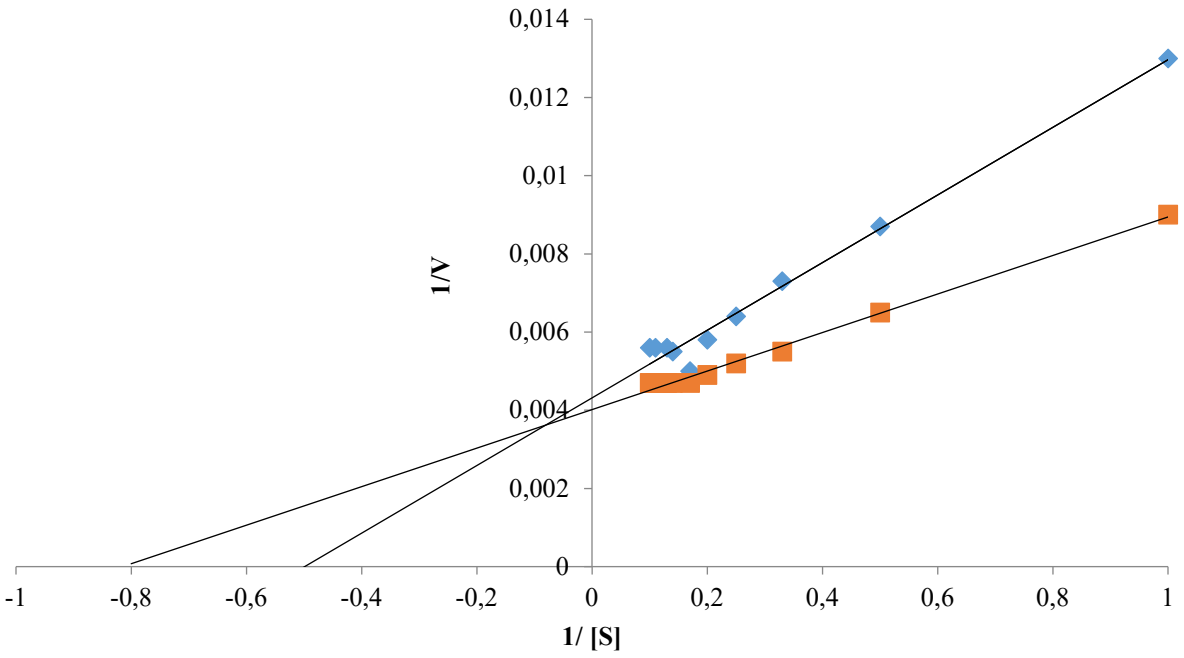

Fig. 6 Lineweaver-Burk plot of free (◆) and immobilized (■) protease from *Bacillus aryabhattai* Ab15-ES. The enzyme activity was measured at various azocasein concentrations (1.0-10.0 mg/ml) at pH 8.0 and 50 °C. The K_m and V_{max} values were determined using Lineweaver-Burk plot. S: Substrate concentration; V: Protease activity.

Storage stability of free and immobilized protease from *Bacillus aryabhattai* Ab15-ES

Storage stability represents a prominent index for the evaluation of properties of enzymes for possible commercialization [73]. Immobilization of enzymes in a suitable support matrix is an important tool for a more rigid molecule that can resist conformational changes [60]. In essence, soluble enzymes are not stable during storage, leading to a reduction in catalytic activity [74]. In the present study, free and immobilized protease were stored separately at 4 and 25 °C for 30 d, and residual activity was determined at 5 d intervals. At 4 °C, the immobilized enzyme was found to be more stable, retaining 80.88% of its original activity after 30 d in comparison to free enzyme that retained 64.6%. However, no significant loss in activity was recorded for free enzyme for up to 5 d (Fig. 7). Remarkably, while the soluble enzyme was almost completely inactivated when stored at 25 °C for 30 d, the immobilized protease still retained 38.37% of its initial activity.

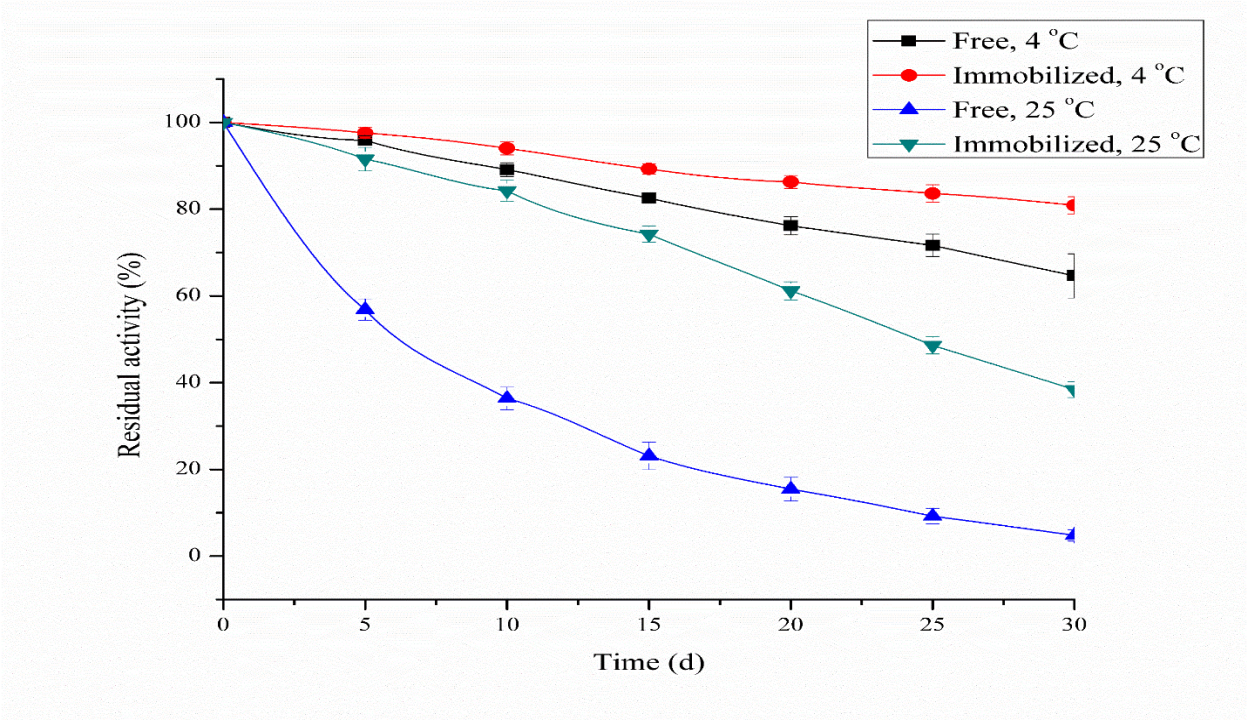

Fig. 7 Storage stability of free and immobilized protease at 4 and 25 °C. Values indicate the average of triplicate values while the error bars represent the standard deviation.

This improved storage stability may be due to reduction in the denaturation of the enzyme or better structural stabilization as a result of immobilization of the enzyme. Similar results have been reported after immobilization of enzymes in alginate gel beads [55,75,76].

Reusability potential of immobilized protease from *Bacillus aryabhattai* Ab15-ES

Reusability of immobilized enzymes is recognized as a crucial factor that influences their cost-effective application for various bioprocesses [66]. In the present study, the reuse potential of immobilized protease was investigated in several cycles for substrate hydrolysis. The immobilized protease retained more than 98% of residual activity after second cycle, and 32.93% of its original activity after continous use for up to seven cycles (Fig. 8), indicating a good operational stability of the biocatalyst.

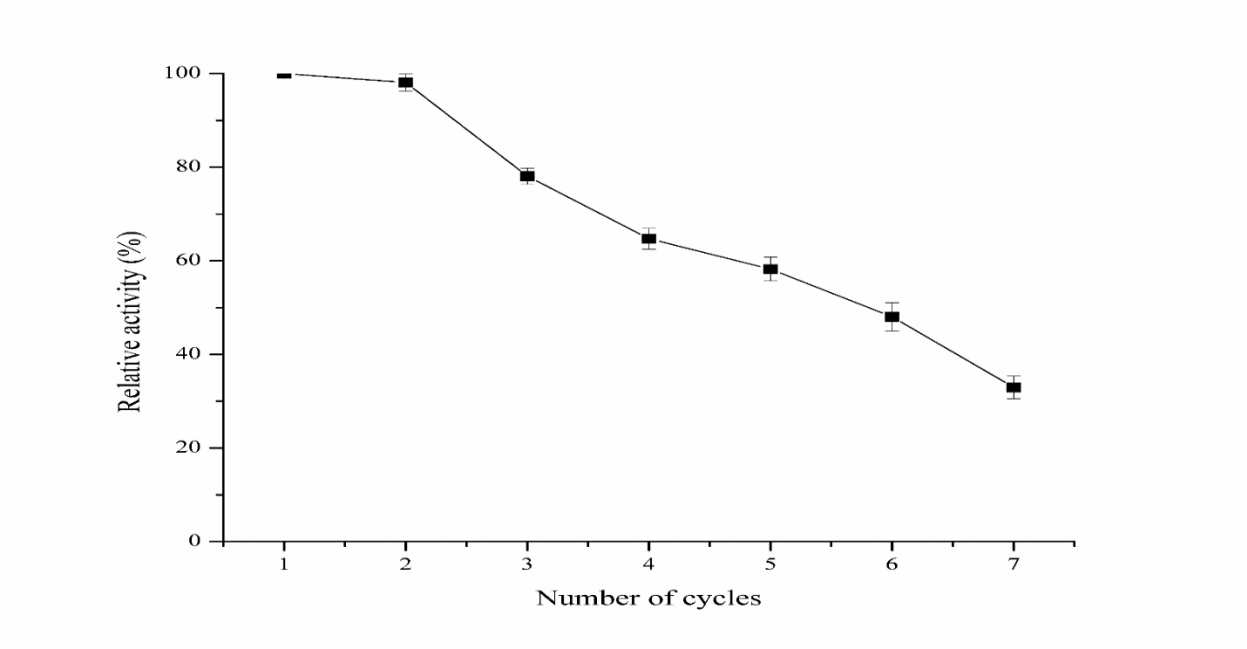

Fig. 8 Reusability of protease immobilized in alginate gel beads. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Repeated use weakens binding strenght between the matrix and the immobilized enzyme resulting in physical loss of the enzyme from the support material. In addition, the loss in the enzyme activity can be linked to the distortion of the active site due to intermittent encounter with the substrate, causing partial or complete loss of catalytic efficiency [60]. Gupta et al. [55] reported on the reusability of immobilized β-glucosidase in alginate beads. The immobilized enzyme was reused four times with a residual activity of 17.85%. Similarly, continous use of protease and urease immobilized in alginate beads for up to three and five cycles, respectively has been reported [20,53,77].

Conclusions

Alkaline protease from *Bacillus aryabhattai* Ab15-ES was successfully immobilized in alginate gel beads, with highest immobilization efficiency obtained at optimum conditions of 2% sodium alginate and 0.3 M calcium chloride. Both free and immobilized protease recorded optimum activity at pH of 8.0 and temperature of 50 °C. Entrapment in alginate gel beads considerably improved thermal and pH stability of the enzyme as well as its storage stability when compared to the soluble enzyme. Furthermore, the immobilized enzyme showed increased affinity towards substrate, higher catalytic efficiency and good operational stability for up to seven reaction cycles, suggesting its significance for sustainable and economic biosynthetic processes.

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Conflicts of interest: The authors declare no conflict of interest

Author contributions: Adegoke Isiaka Adetunji designed and conducted the experiments; analyzed the data and drafted the manuscript while Ademola Olufolahan Olaniran conceptualized and designed the experiment as well as edited the manuscript.

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CHAPTER NINE

LIPASE FROM AN INDIGENOUS *BACILLUS ARYABHATTAI* SE3-PB: PARTIAL PURIFICATION, IMMOBILIZATION AND BIOCHEMICAL PROPERTIES

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Lipase from an indigenous *Bacillus aryabhattai* SE3-PB: partial purification, immobilization and biochemical properties

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Abstract

Extracellular lipase from an indigenous *Bacillus aryabhattai* SE3-PB was partially purified by ammonium sulphate precipitation, and then immobilized in alginate beads by entrapment method. Biochemical properties of both free and immobilized lipases were further investigated. After optimization of immobilization conditions, maximum immobilization efficiencies of 77 ± 1.53% and 75.99 ± 3.49% were recorded at optimum concentrations of 2% (w/v) sodium alginate and 0.2 M calcium chloride, respectively for the entrapped enzyme. There was no change in the optimum temperature and pH of both enzyme preparations, with maximum activity attained at 60 °C and 9.5, respectively. In comparison to free lipase, the entrapped enzyme demonstrated improved stability over the studied pH range (8.5-9.5) and temperature (55-65 °C) when incubated for 3 h. Furthermore, the immobilized lipase showed enhanced enzyme-substrate affinity and higher catalytic efficiency when compared to soluble enzyme. The entrapped enzyme was also found to be more stable, retaining 61.51 and 49.44% of its original activity after being stored for 30 d at 4 and 25 °C, respectively. In addition, the insolubilized enzyme exhibited good reusability with 18.46% relative activity after being repeatedly used for six times. These findings suggest the efficacy of the prepared immobilized lipase for various biotechnological applications.

Keywords *Bacillus aryabhattai* SE3-PB; Lipase; Immobilization; Alginate beads; Biotechnological applications

Introduction

Lipases (EC 3.1.1.3; triacylglycerol acylhydrolases) are biocatalysts that involved in the hydrolysis of ester bonds present in fats and oils into di-glycerides, mono-glycerides, glycerol and free fatty acids at the oil-water interface (Treichel et al. 2010). In addition, lipases also catalyze synthesis reactions including esterification, trans-esterification, inter-esterification, alcoholysis, acidolysis, and aminolysis in non-aqueous and micro-aqueous milieu (Casas-Godoy et al. 2012; Kanmani et al. 2015). The potential of lipases to catalyze different reactions using wide range of substrates in the absence of expensive cofactors coupled with high activity and stability in organic solvents led to lipases being grouped as third largest class of commercialized enzymes after proteases and carbohydrases (Hasan et al. 2006; Casas-Godoy et al. 2012). Lipases are produced by numerous species of animals, plants, bacteria, and fungi (Dutta and Ray 2009; Nagarajan 2012; Kanmani et al. 2015). However, lipase of microbial origin have gained vast attraction owing to their good stability, substrate specificity, ease of genetic manipulation, lower production cost and higher productivity at shorter generation time (Hasan et al. 2006). Lipases are commonly used in food, pulp

and paper, detergent, tannery, textile, pharmaceutical, agrochemical and cosmetic industries as well as in treatment of oil-contaminated wastewater (Hasan et al. 2006; Sangeetha et al. 2011).

Free enzymes are generally soluble in homogenous catalysis system, leading to product contamination and eliminating their recovery for reuse in the active form from most of the reaction mixtures, thus can only be used once in solutions (Homaei et al. 2013). Moreover, free enzymes are not only unstable and inactivated by different environmental conditions (ionic strength, temperature, pH, inhibitors), but too costly for biotechnological applications (Guisan 2006; Lee et al. 2010; Hernandez and Fernandez-Lafuente 2011; Sangeetha et al. 2011). In order to abate the aforementioned challenges and make enzyme utilization in bioprocesses more favorable, immobilization has been recognized as an important strategy in enzyme technology.

Enzyme immobilization involves retention of catalytic activity of the enzyme in a certain defined region in order to permit its reusability (Mohamad et al. 2015). It offers several advantages including increased activity and stability over broader range of temperature and pH, easier product recovery, cost reduction, recovery yield, possibility of continuous process, rapid termination of reactions, controlled product formation and adaptability to a variety of configurations and specific processes in reactors (Spahn and Minteer 2008; Homaei et al. 2013).

The choice of appropriate immobilization technique is key to a successful immobilization process, as it plays a paramount role in determining the enzyme activity and characteristics in a particular reaction (Mohamad et al. 2015). Effectiveness of each technique depends on reaction conditions, process of product formation and cost evaluation (Bhushan et al. 2015). Methods such as physical adsorption, entrapment, covalent binding and cross-linking are employed for lipase immobilization (Minovska et al. 2005; Fernandez-Lafuente 2010). However, entrapment in appropriate support material is known as the most efficient technique due to its ability to enhance mechanical stability, minimize enzyme leaching, and prevent chemical interaction of the enzyme with the support material (Shen et al. 2011). In addition, entrapment permits modification of encapsulating material and create optimal microenvironment for the enzyme (Mohamad et al. 2015). Such microenvironment can be achieved with a range of support materials (Mendes et al. 2011; Datta et al. 2013; Santos et al. 2015).

Gelation of polyanionic or polycationic polymers (e.g. alginate) in the presence of counter-ions represents an easy and commonly employed approach for enzyme entrapment. However, alginate is by far the most widely used polymer for immobilization and microencapsulation technologies due to its mild gelling properties, non-toxicity, biocompatibility, thermostability, ease of formulation, low cost, effective particle size and availability (Won et al. 2005; Yadav et al. 2012). Alginate is an anionic linear heteropolysaccharide

extracted from marine brown algae and seaweeds, and consist of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid residues arranged sequentially in different proportions. Polyvalent cations such as calcium chloride bind to the biopolymer whenever there are two neighboring guluronic acid residues forming an ionotropic gelation of alginate (Buchholz et al. 2012). Lipase immobilization by entrapment using alginate beads has been reported (Shafei and Allam 2010; Bonine et al. 2014; Zhang et al. 2013, 2014).

Lipases are serine hydrolases consisting of consensus sequence G-X_1- S-X_2-G as the catalytic moiety around the active site serine, where G = glycine, S = serine, X_1 = histidine, X_2 = glutamic or aspartic acid (Jaeger and Reetz 1998). The active site of lipase is covered by a lid-like α-helical structure. The lid moves away upon binding to a lipid interface, causing the active site of lipase fully accessible, thus promoting hydrophobic interaction of the enzyme and lipid surface (Jaeger and Reetz 1998). The three-dimensional structure of lipase revealed the presence of a characteristic (α/β) hydrolase fold (Carr and Ollis 2009). When act on emulsified substrates, lipases showed interfacial activation and possess different kind of selectivity towards substrates. These include chemo-specificity, regio-specificity, and enantio-selectivity (Villeneuve 2003; Borrelli and Trono 2015). Characterization of lipase determines its appropriateness for various environmental and industrial applications. Parameters such as pH, temperature, organic solvents, metal ions, detergents/surfactants, and other inhibitors enhance or suppress lipase activity (Hasan et al. 2009).

Owing to the versatility and specific activity of lipase to catalyze wide range of bioconversion reactions, the need arise to bioprospect for indigenous bacterial strains with robust catalytic efficiency. This study therefore focused on partial purification of lipase produced by *Bacillus aryabhattai* SE3-PB isolated from lipid-rich wastewater. Subsequently, comparative study of biochemical properties of the free and immobilized lipases was investigated in order to establish their possible biotechnological applications.

Materials and methods

Partial purification of lipase

Bacillus aryabhattai SE3-PB was grown in optimized medium consisting (in %, w/v, v/v): peptone 0.5, yeast extract 0.5, NaCl 0.05, $CaCl_2$ 0.005, sunflower oil 2 and pH was adjusted to 7.6. The flasks were incubated at 40 °C for 48 h at 193 rpm. The cell-free culture supernatant regarded as crude enzyme was precipitated by ammonium sulphate at 80% saturation. Precipitates were harvested by centrifugation at 10000 rpm for 10 min at 4 ºC, resuspended in a minimal amount of 0.05 M Tris-HCl buffer (pH 7.2) to allow solubilization of proteins and then dialyzed against the same buffer at 4 ℃ overnight to remove residual ammonium sulphate. The lipase activity and protein content of the partially purified enzyme were determined.

Immobilization of partially purified lipase in alginate gel beads

Varying concentrations (1-5%) of sodium alginate were prepared by adding different quantities to 0.05 M Tris-HCl buffer (pH 7.2). Partially purified lipase was immobilized by mixing in equal volume (1:1) with sodium alginate solution by continuous stirring to obtain homogenous suspension. The enzyme-alginate mixture was added dropwise to 5 ml of $CaCl_2$ solution (0.1-0.5 M) from a 5 cm height using a hypodermic syringe. The beads were kept for curing at 4 °C for 1 h (Bonine et al. 2014). Beads of about 3 mm diameter were collected from the solution by filtration and then washed 3-4 times under mild agitation for 15 min with Tris-HCl buffer (0.05 M, pH 7.2) and distilled water to remove unbound enzyme (Won et al. 2005). Similar approach was used for the preparation of control calcium alginate beads without the enzyme.

Lipase assay

Lipase activity was determined as described by Winkler and Stuckmann (1979) with some modifications using *p*-NPP as a substrate. A quantity of 30 mg of *p*-NPP was dissolved in 10 ml isopropanol, mixed with 90 ml of 0.05 M Tris-HCl buffer (pH 7.2) containing 207 mg of sodium deoxycholate and 100 mg of gum arabic. A 2.4 ml freshly prepared substrate solution was pre-warmed at 37 ºC and then mixed with 100 µl of the enzyme solution. The reaction mixture was further incubated at 37 ºC for 15 min. For the immobilized enzyme, 0.6 g of microspheres was used. Absorbance was measured against the enzyme-free blank at 410 nm using UV-Visible spectrophotometer (UVmini-1240, Schimadzu, Australia). Total protein estimation was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard. One unit of lipase activity was defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol per ml min^{-1} under standard assay conditions. The assays were carried out in triplicate.

Determination of immobilization efficiency

The immobilization efficiency, *Y* is described as the percentage of bound enzyme activity observed in the immobilizate, and is illustrated in the eq. (1) (Sheldon and van Pelt 2013):

$$Y\ (\%) = \frac{\text{Observed activity}}{\text{Immobilized activity}} X\ 100 \qquad (1)$$

Where immobilized activity was determined by measuring the total residual enzyme activity that remains in the enzyme solution after immobilization and subtracting this from the total starting activity (Sheldon and van Pelt 2013).

Biochemical properties of free and immobilized lipase

Effect of temperature and pH on the activity of free and immobilized lipase from Bacillus aryabhattai SE3-PB

The influence of temperature on activity of free and immobilized lipase was assessed by carrying out enzyme assay at temperatures ranging between 30-80 ºC at pH 7.2. Lipase activity was measured according to a standard protocol using *p*-NPP as a substrate. The optimum pH of free and immobilized lipase activity was determined by carrying out enzyme assay at optimum temperature for 15 min using *p*-NPP as a substrate in buffer solutions of pH values ranging from 3.0-11.0. The buffers used include 0.05 M citrate buffer (pH 3.0-4.5), 0.05 M acetate buffer (pH 5.0-5.5), 0.05 M phosphate buffer (pH 6.0-7.0), 0.05 M Tris-HCl buffer (pH 7.5-9.0) and 0.05 M Glycine-NaOH buffer (pH 9.5-11.0). The relative lipase activities were calculated as percentage (%) of maxiumum enzyme activity and the values were plotted against the respective temperature or pH. All experiments were done in triplicate.

Effect of temperature and pH on the stability of free and immobilized lipase from Bacillus aryabhattai SE3-PB

The thermal stability of free and immobilized lipase was determined by pre-incubating the enzyme samples in 0.05 M Tris-HCl buffer (pH 9.5) for up to 3 h at 55, 60 and 65 °C. Aliquots were withdrawn at 30 min intervals and assayed for residual enzyme activity using the standard *p*-NPP method. The non-heated enzyme was considered as a control (100%). The pH stability of the free and immobilized lipases was investigated by pre-incubating the enzyme samples in respective buffers (pH 8.5, 9.0 and 9.5) at optimum temperature for up to 3 h. Aliquots were withdrawn at 30 min intervals. Residual lipase activity was measured under standard assay conditions and calculated against the initial enzyme activity (100%). All experiments were carried out in triplicate.

Kinetics properties of free and immobilized lipase

Lipase assay was carried out in 0.05 M reaction buffer (Tris-HCl, pH 9.5) with varying concentrations (0.1-50 mM) of *p*-NPP. The reaction mixture was incubated at 60 ºC for 15 min. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were calculated from Lineweaver-Burk plot.

Storage stability of free and immobilized lipase from Bacillus aryabhattai SE3-PB

The storage stability of free and entrapped lipase was determined by preserving the enzyme at 4 and 25 ºC for 30 d. Enzyme samples were taken at 5-d intervals and assayed for residual activity in the presence of *p*-

NPP as a substrate under standard conditions. Residual activity was calculated with reference to the initial activity (100%). All experiments were done in triplicate.

Reusability potential of immobilized lipase from Bacillus aryabhattai SE3-PB

The reusability of the entrapped lipase was assessed by repeating the enzyme assay several times at 37 °C for 15 min. Thereafter, the beads were separated, washed with Tris-HCl buffer (pH 7.2) and re-introduced into the reaction medium consisting of fresh substrate solution. Lipase activity was measured after every reaction time under standard assay conditions. The relative activity was calculated by taking the enzyme activity of the freshly prepared beads in the first run as 100%. All experiments were done in triplicate.

Scanning electron microscopy of beads with immobilized lipase

The morphological characterization of the beads with immobilized lipase was studied using ZEISS EVO LS15 scanning electron microscope (SEM).

Results and discussion

Partial purification of lipase

The extracellular lipase from *Bacillus aryabhattai* SE3-PB was partially purified by ammonium sulphate precipitation up to a saturation of 0-80% followed by dialysis at 4 °C. Lipase activity and total protein of the resulting dialysate were determined. A 30.84% recovery was recorded with a purification of 2.11-fold and a specific activity of 144.76 U/mg (Table 1). Ammonium sulphate precipitation of crude lipase from *Pseudomonas fluorescens* RB02-3 has been reported (Boran and Ugur 2010). Results showed a purification fold of 2.19 with a specific activity and recovery of 115.30 U/mg and 21.5%, respectively.

Table 1 Profile of purification of lipase from *Bacillus aryabhattai* SE3-PB

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme	26402	385.51	68.49	100	1.00
$(NH_4)_2SO_4$ precipitation (80%)	8143	56.25	144.76	30.84	2.11

Lipase immobilization and scanning electron microscopic analysis

Since cross-linking between alginate and calcium chloride results in gelation, the concentrations of alginate and calcium chloride formed a key factor for enzyme immobilization. Therefore, in the present study, influence of alginate concentrations (1-5%, w/v) and 0.2 M calcium chloride on immobilization efficiency of lipase from *Bacillus aryabhattai* SE3-PB was investigated. There was an increase in immobilization efficiency as the concentration of alginate increases from 1-2% (w/v), with maximum immobilization efficiency (77 ± 1.53%) recorded from the beads prepared from 2% alginate. After which, the immobilization efficiency decreases, lowest immobilization efficiency of 34.49 ± 1.01% was obtained from 1% alginate (Table 2).

Table 2 Immobilization efficiency of lipase from *Bacillus aryabhattai* SE3-PB at varying sodium alginate concentrations

Sodium alginate concentration (%)	Immobilization efficiency (%)
1	34.49 ± 1.01
2	77 ± 1.53
3	59.4 ± 2.69
4	42.27 ± 2.08
5	37.47 ± 2.12

*All values are expressed as mean from triplicate values; ± indicates SD

The decrease in immobilization efficiency as the alginate concentration increases may probably be due to porosity of the support material, causing diffusion limitation of the substrate transfer from the bulk phase into the alginate beads (Knezevic et al. 2002). Maximum immobilization efficiency of enzymes has been reported from 2% alginate (Ahmed et al. 2008; Demirkan et al. 2011). Furthermore, alginate concentrations in the range of 1-3% (w/v) have been reported for maximum immobilization efficiency of lipase (Bhushan et al. 2008; Bonine et al. 2014; Kumar et al. 2014).

The concentration of calcium chloride has a significant effect on the stability and pore size of the beads. In order to determine its optimum concentration for maximum activity of the entrapped enzyme, alginate beads were prepared at various calcium chloride concentrations ranging from 0.1 to 0.5 M using 2% sodium alginate. Maximum immobilization efficiency (75.99 ± 3.49%) was recorded from beads prepared from 0.2 M calcium chloride. Further increase in calcium chloride concentration gave rise to decrease in

immobilization efficiency (Table 3). This supports the findings of Jobanputra et al. (2011) in which maximum entrapped enzyme activity was recorded from beads prepared from 0.2 M calcium chloride.

Table 3 Immobilization efficiency of lipase from *Bacillus aryabhattai* Ab15-ES at varying calcium chloride concentrations

Calcium chloride concentration (M)	Immobilization efficiency (%)
0.1	48.67 ± 1.13
0.2	75.99 ± 3.49
0.3	62.35 ± 2.97
0.4	46.75 ± 0.96
0.5	38.66 ± 0.58

*All values are expressed as mean from triplicate values; ± indicates SD

The structural features of alginate beads prepared by physical methods have been extensively studied by many researchers (De Queiroz et al. 2006). SEM analysis shows the formation of pores on the bead surface, and microspheres, which create microenvironment for enhanced enzyme-substrate reaction (Figure 1). Zhan et al. (2013) reported similar findings during characterization of phospholipase A1 immobilized in polyvinyl alcohol-alginate matrix using SEM.

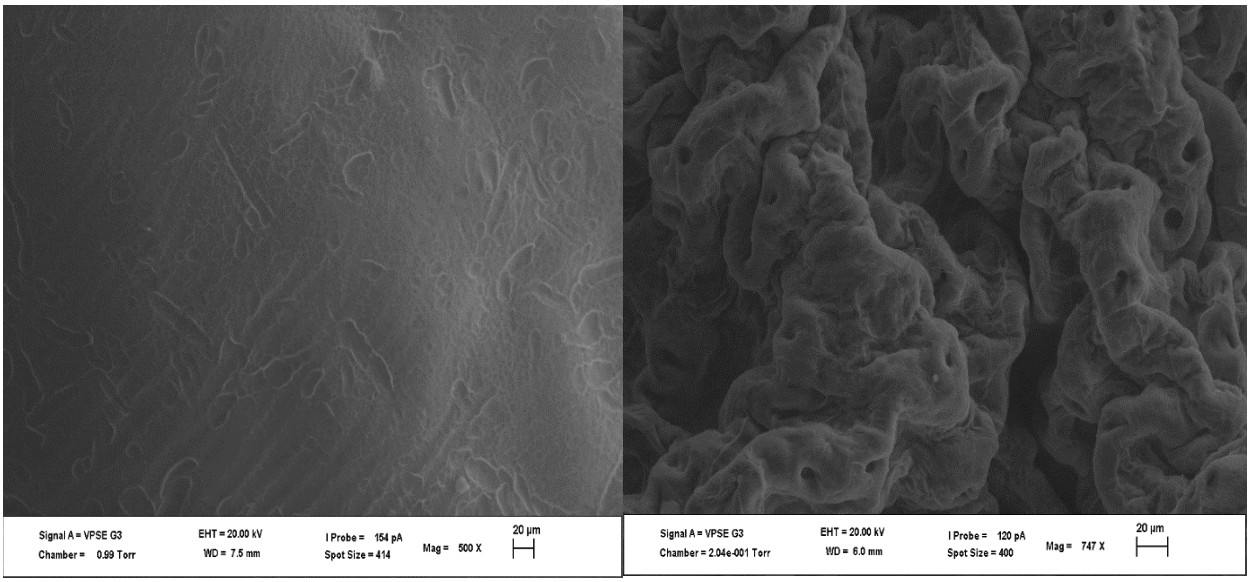

Fig. 1 SEM image of morphology of alginate gel beads

Biochemical properties of free and immobilized lipase

Effect of temperature and pH on the activity of free and immobilized lipase from Bacillus aryabhattai SE3-PB

The relative activity profile of free and immobilized lipase from *Bacillus aryabhattai* SE3-PB was determined by incubating the enzyme and substrate solution (pH 7.2) at temperatures ranging from 30-80 ºC. There was an increase in relative activity of both enzyme preparations as the temperature increases from 30-60 °C, with maximum activity recorded at 60 ºC for free and entrapped lipase (Figure 2). Optimum temperature in the range of 50-60 °C for soluble and immobilized lipase in solid matrices has been reported (Nawani et al. 2006; Zhan et al. 2013; He et al. 2017). Beyond 60 ºC, the activity decreased up to 80 ºC. However, the immobilized lipase recorded higher relative activity at a wider range of temperature than the free enzyme, with more than 60% activity maintained between 65-80 ºC.

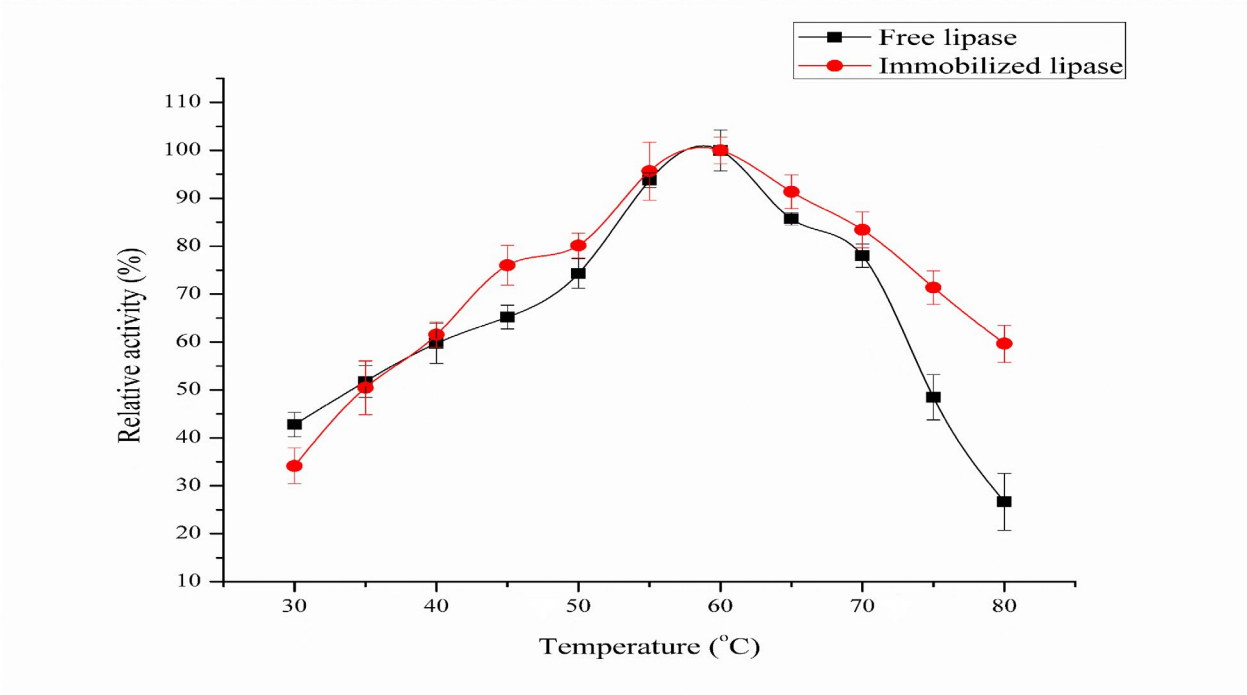

Fig. 2 Effect of temperature on the activity of free and immobilized lipase from *Bacillus aryabhattai* SE3-PB. Lipase activity was measured under standard assay conditions at various temperatures (30-80 °C) at pH 7.2. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Further increase in temperature beyond optimum level caused a sharp reduction in relative activity of the free lipase, reaching 26.65% at 80 °C. In contrast, the immobilized enzyme maintained approximately 60% of its relative activity, found to be 2.24-fold higher than that of the free enzyme, an indication of better heat resistance of the enzyme after immobilization in alginate beads. The low relative activity of immobilized lipase at 30 ºC in comparison to that of free enzyme may be due to steric hindrance of the enzyme active site by the support material, or less of enzyme flexibility for substrate binding.

The influence of pH on the relative activity of free and immobilized lipase was studied using *p*-NPP in the pH range of 3.0-11.0 at 37 °C for 15 min (Figure 3). Both enzyme preparations showed considerable activity in the broad pH range of 5.0-10.0, with maximum relative activity observed at pH 9.5, suggesting alkaline nature of the enzyme. Further increase in pH beyond optimum resulted in a rapid decrease in relative activity of both soluble and entrapped enzyme. This may probably be due to change in pH, which affects the protein structure and enzyme activity (Ohnishi et al. 1994). Interestingly, at pH beyond optimum, particularly at pH 11.0, the immobilized lipase exhibited higher relative activity (55.53%) in comparison to the free enzyme (30.3%). This clearly indicated resistance of the immobilized enzyme to extreme alkaline medium that results in its inactivation.

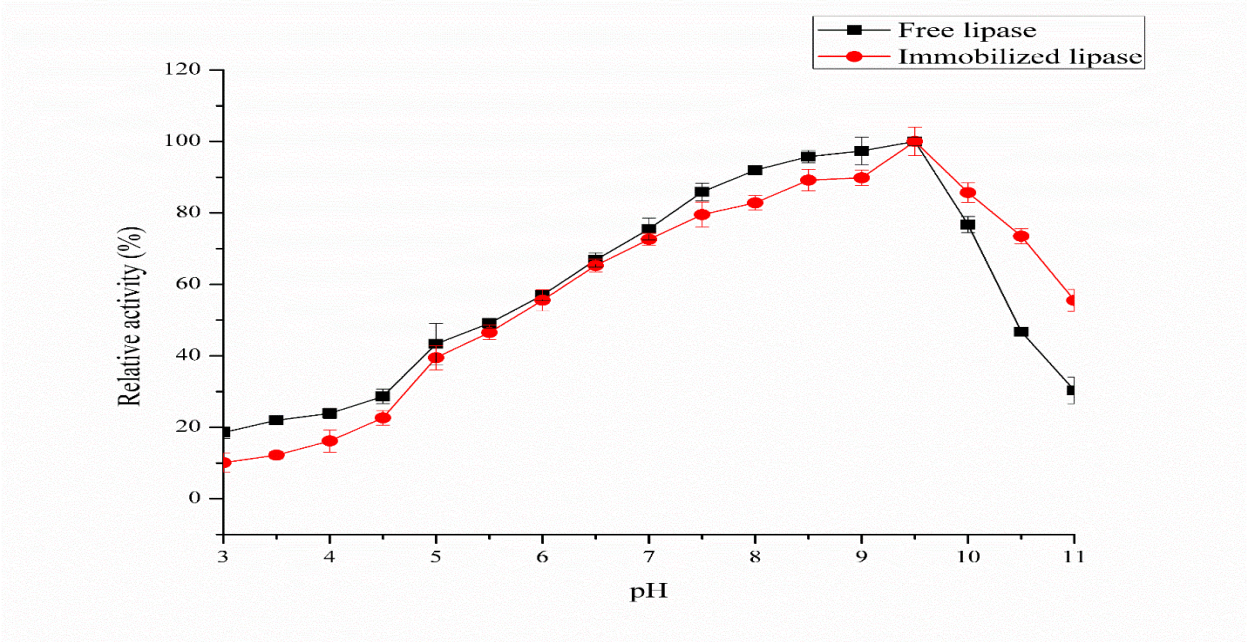

Fig. 3 Effect of pH on the activity of free and immobilized lipase from *Bacillus aryabhattai* SE3-PB. Lipase activity was determined at 60 °C. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Effect of temperature and pH on the stability of free and immobilized lipase from Bacillus aryabhattai SE3-PB

The thermal stability of free and immobilized lipase was assessed by pre-incubating both enzyme preparations at various temperatures (55-65 °C) for 3 h. As illustrated in Figure 4, for all the tested conditions, there was a decrease in residual activity as the temperature increased. The immobilized lipase retained 73.65% of its activity after 3 h of incubation at 55 °C, while that of the free enzyme was 61.89%.

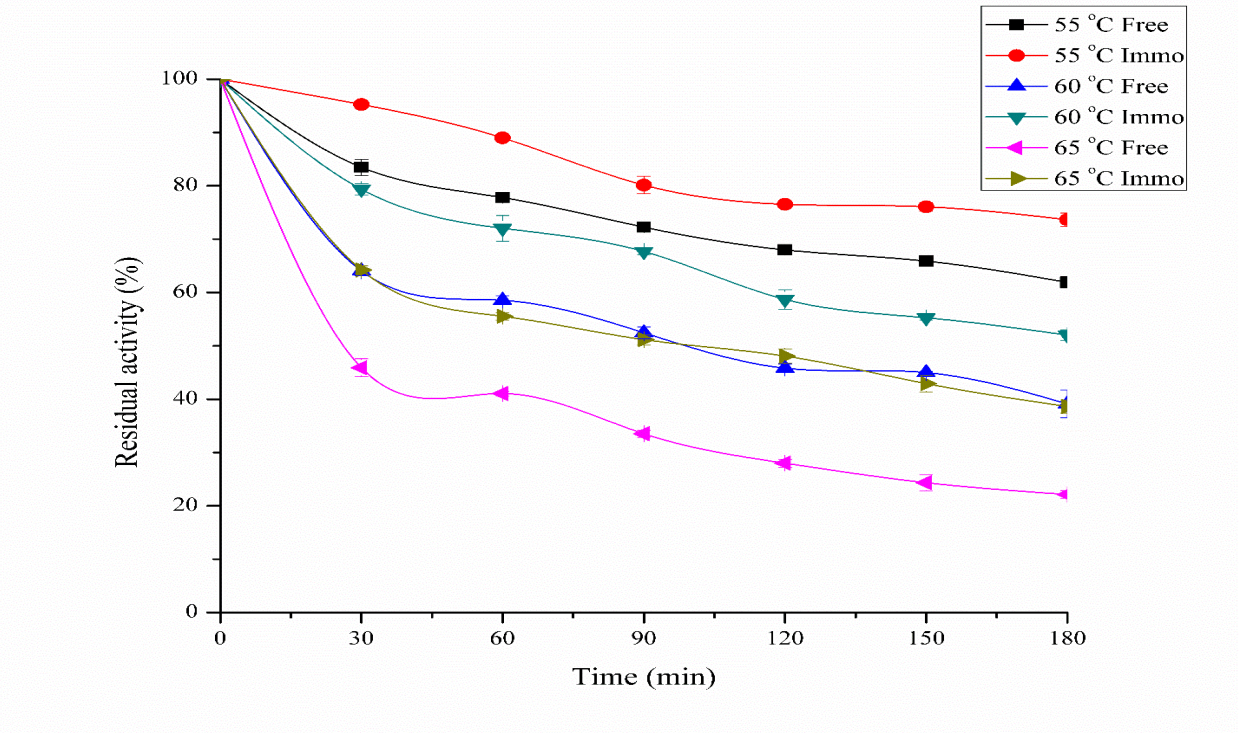

Fig. 4 Thermal stability of free and immobilized lipase from *Bacillus aryabhattai* SE3-PB. The enzyme was pre-incubated at different temperatures (55-65 °C) for 3 h at pH 9.5, and the residual enzyme activities were estimated at regular intervals under standard assay conditions. The non-heated enzymes were taken as 100%. Values indicate the average of triplicate values while the error bars represent the standard deviation.

When the incubation temperature was increased to 65 °C for 3 h, the entrapped enzyme retained 38.61% of its original activity, while the residual activity of the free enzyme was 22.12%. The immobilized lipase was much more stable perhaps due to enhanced rigidity and stability of the secondary structure of the enzyme after immobilization (Mahmoud et al. 2013). When comparing the activities of free and immobilized lipase, immobilization led to a considerably higher thermal stability, suggesting its importance for various biotechnological applications (Cao et al. 2016). Increased thermal stability of lipase entrapped in alginate

beads has been reported (Bhushan et al. 2008; Shafei and Allam 2010; Bonine et al. 2014; Kumar et al. 2014).

The pH stability of free and immobilized lipase in alginate beads was investigated by pre-incubating the enzyme in 0.05 M of respective buffers (pH 8.5-9.5) at the optimum temperature for 3 h. Samples were taken at regular intervals and assayed for residual activity. Results obtained showed no significant loss in activity at pH 8.5 by the immobilized enzyme after incubation for 3 h whereas, the soluble enzyme retained only 93.75% of its activity (Figure 5). The immobilized lipase retained 63.84 and 57.36% of its activity at pH 9.0 and 9.5, respectively whereas, the free enzyme maintained 56.04 and 45.44%, respectively of its initial activity when incubated for 3 h. These findings showed that the conformation of lipase immobilized in alginate beads was more stable, resulting in a broad pH tolerance.

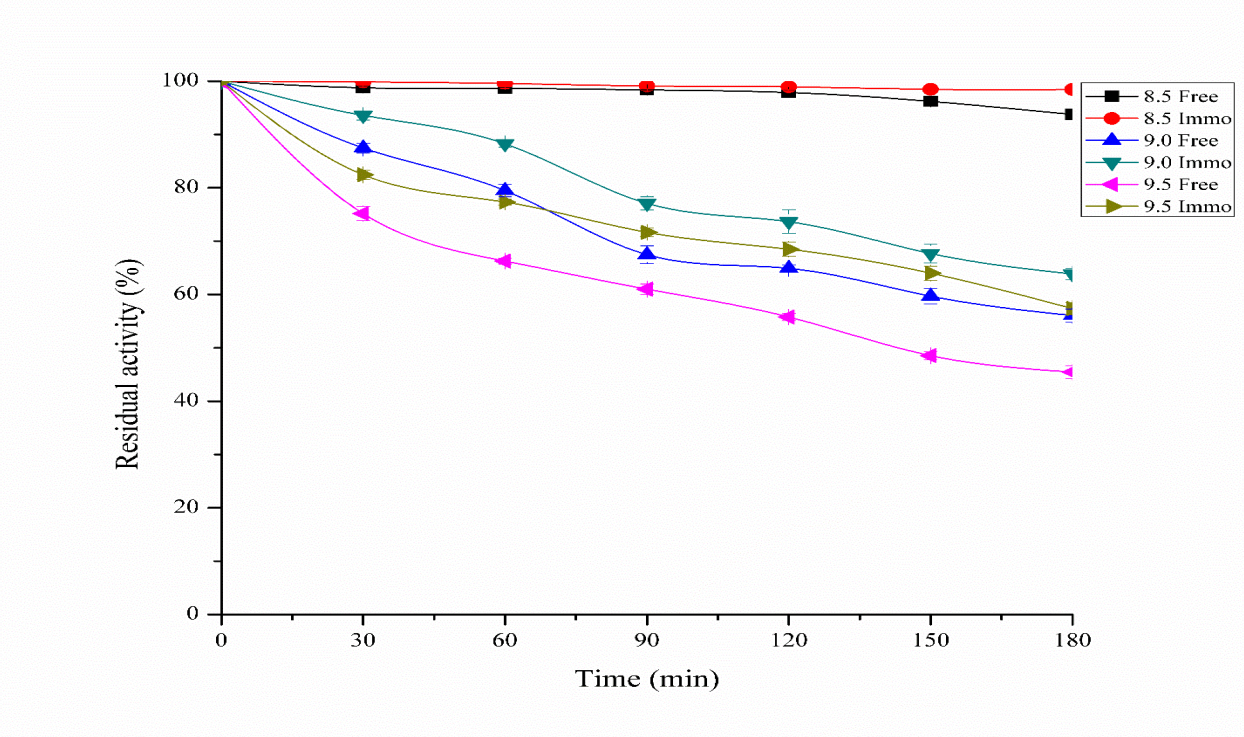

Fig. 5 pH stability of free and immobilized lipase from *Bacillus aryabhattai* SE3-PB. The enzyme was pre-incubated at pH values (8.5-9.5) for 3 h at 60 °C, and the residual enzyme activities were estimated at regular intervals under standard assay conditions. The non-heated enzymes were taken as 100%. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Kinetics parameters of free and immobilized lipase

The kinetics parameters of hydrolysis of *p*-NPP by free and immobilized lipase were determined at various substrate concentrations (0.1-50 mM). The Michaelis-Menten constants (K_m and V_{max}) for both free and immobilized enzyme were calculated from Lineweaver-Burk plot (Figure 6). The immobilized lipase in alginate beads recorded lower K_m value (0.1304 mM) in comparison to the free counterpart (0.1509 mM), an indication of enhanced enzyme-substrate affinity after immobilization. Several authors have reported similar results in literature (Kumar et al. 2014; Cao et al. 2016). In addition, the V_{max} (217.39 U/ml) of the immobilized lipase was found to be higher than that of free enzyme, suggesting increased accessibility of substrates to the active sites of the enzyme. Moreover, the V_{max}/K_m value for immobilized lipase (1667.10) was higher than the corresponding value for free enzyme (1250.36), demonstrating better attachment of the immobilized enzyme to the substrate and high catalytic efficiency.

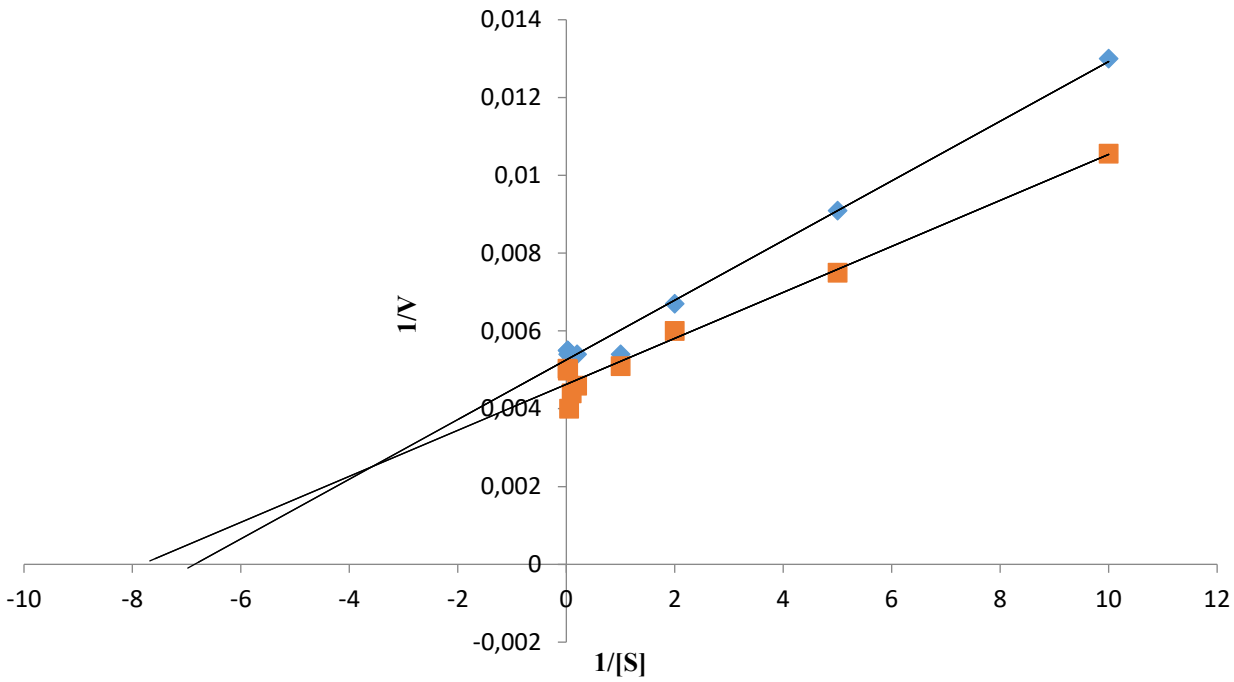

Fig. 6 Lineweaver-Burk plot of free (◆) and immobilized (■) lipase from *Bacillus aryabhattai* SE3-PB. The enzyme activity was measured at various *p*-NPP concentrations (0.1-50 mM) at pH 9.5 and 60 °C. The K_m and V_{max} values were determined using Lineweaver-Burk plot. S: Substrate concentration; V: Protease activity.

Storage stability of free and immobilized lipase from Bacillus aryabhattai SE3-PB

The storage stability of free and immobilized lipase was determined by preserving both enzyme preparations at 4 and 25 °C for 30 d. Samples were taken at 5-d intervals and assayed for residual activity. As shown in Figure 7, the immobilized lipase was more stable, retaining higher activity in comparison to the free enzyme. At 4 and 25 °C, lipase entrapped in alginate beads maintained 61.51 and 49.44%, respectively of its initial activities after 30 d of storage, while the corresponding values for the free enzyme were 43.92 and 4.93%, respectively. This explained the modification of the three-dimensional structure of the enzyme after immobilization (Yilmaz et al. 2011). The loss in the residual activity of the free lipase during storage might be due to its increased susceptibility to autolysis (He et al. 2017). This corroborates similar findings previously reported for lipase immobilized in various solid matrices (Huang et al. 2011; Cao et al. 2016; He et al. 2017).

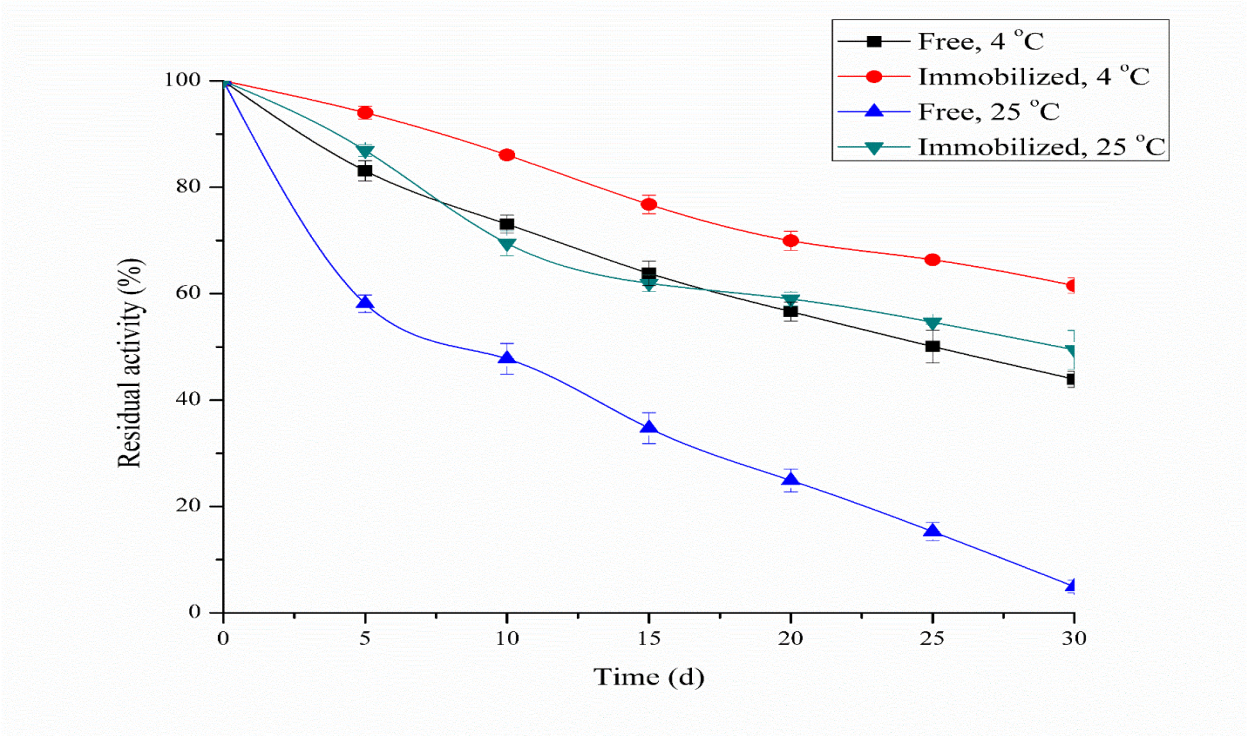

Fig. 7 Storage stability of free and immobilized lipase at 4 and 25 °C. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Reusability potential of immobilized lipase from Bacillus aryabhattai SE3-PB

Among the most important advantages of immobilized enzyme is reusability, which can effectively reduce its operational costs for large-scale applications. In the present study, lipase immobilized in alginate gel beads was used continuously for enzymatic hydrolysis of *p*-NPP under standard assay conditions. As shown in Figure 8, the relative activity of the immobilized lipase reduced with increase in the number of reuses. The immobilized enzyme recorded a relative activity of 18.46% after six cycles. The decrease in activity may be due to physical loss or leakage of the enzyme from the beads. Such activity loss from reuse of lipase entrapped in alginate beads has been reported (Won et al. 2005). This supports the findings of Zhang et al. (2013) and Bonine et al. (2014) in which lipase immobilized in alginate beads was used for six cycles.

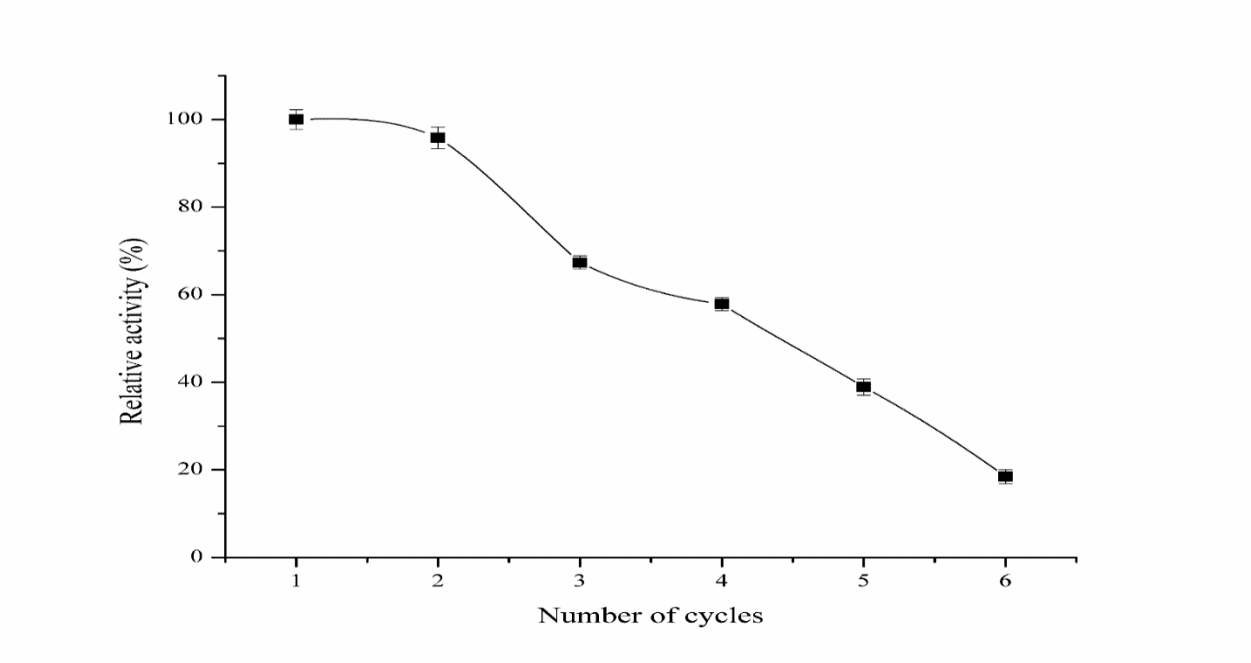

Fig. 8 Reusability of lipase immobilized in alginate gel beads. Values indicate the average of triplicate values while the error bars represent the standard deviation.

Conclusions

In the present study, calcium alginate gel beads were prepared and used as support material for immobilization of lipase from *Bacillus aryabhattai* SE3-PB via entrapment method. Maximum entrapped enzyme activity was recorded at optimal conditions of 2% (w/v) sodium alginate and 0.2 M calcium

chloride. Both free and immobilized lipase had maximum activity at optimum temperature and pH of 60 °C and 9.5, respectively. However, the entrapped enzyme exhibited improved pH, temperature and storage stability as well as enhanced affinity for the substrate and higher catalytic efficiency when compred to the soluble counterpart. Furthermore, the entrapped lipase showed better reusability for up to six consecutive cycles during repeated hydrolysis of *p*-NPP, suggesting the efficient, longevity and cost-effectiveness of the developed immobilized biocatalyst for various biotechnological applications.

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CHAPTER TEN

TREATMENT OF LIPID-RICH WASTEWATER USING A MIXTURE OF FREE OR IMMOBILIZED BIOEMULSIFIER AND HYDROLYTIC ENZYMES FROM INDIGENOUS BACTERIAL ISOLATES

This chapter has been submitted in the current format to the 'Desalination and Water Treatment'

Treatment of lipid-rich wastewater using a mixture of free or immobilized bioemulsifier and hydrolytic enzymes from indigenous bacterial isolates

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Abstract

The combined use of free or immobilized bioemulsifier produced by *Acinetobacter* sp. and enzyme pool (protease and lipase) from *Bacillus aryabhattai* in the reduction of organic pollutants present in lipid-rich wastewater was investigated. Physicochemical characterization of the raw wastewater revealed high pollutant load in poultry processing wastewater in comparison to dairy wastewater. Biodegradability of the wastewater was assessed by measuring the reduction of COD and lipid content at time intervals under varying incubation conditions. In dairy wastewater treated at 37 °C without pH adjustment, maximum COD (60.51 & 65.19%) and lipid (47.98 & 63.53%) reduction efficiencies were recorded at 120 h using free and immobilized bioproducts, respectively. However, under these conditions, maximum COD (86.44 & 93.65%) and lipid (51.62 & 69.06%) removal efficiencies of poultry processing wastewater were observed at 120 h when treated with free and immobilized bioproducts, respectively. At temperature of 50 °C and pH 8.0, there was improved reduction of organic pollutants, with maximum COD (65.96 & 77.52%) and lipid (55.22 & 71.12%) removal efficiencies obtained in dairy wastewater at 72 h when using free and immobilized bioproducts, respectively. In the case of poultry processing wastewater, optimum COD (90.29 & 94.72%) and lipid (63 & 76.66%) removal was recorded at 72 h when treated with free and immobilized bioproducts, respectively. Reusability studies suggest that the immobilized bioproducts could be reused for up to six and seven times for the treatment of dairy and poultry processing wastewaters, respectively. This study suggests the efficient and synergistic application of the developed immobilized bioemulsifier and hydrolytic enzymes in the treatment of high fat-containing wastewater.

Keywords: Lipid-rich wastewater; Bioproducts; Biodegradation; Organic pollutants; Hydrolytic enzymes

1. Introduction

Lipids (fats, oils and greases) and proteins are among the most indispensable components of natural foods as well as many synthetic compounds and emulsions [1,2]. They constitute a vital constituent of wastewater discharged from restaurants, oil mill industries, dairy industries and slaughterhouses [3,4]. These industries utilize large amounts of water for various processes, resulting in significant effluent volume with huge amounts of organic matter [5]. In addition, this wastewater is characterized by high concentrations of chemical oxygen demand (COD), biochemical oxygen demand (BOD), suspended solids along with many toxic compounds, causing severe environmental pollution [6,7]. These include formation of oil films on water surfaces, which prevent transfer of dissolved oxygen into aerobic process. Aggregates of oil droplets caused blockage of sewer lines, resulting in unpleasant odor; flotation, mass transfer problems for soluble substrates, and reduction of sludge acetogenic and methanogenic activities [8-10]. Therefore, effective techniques to lessen the negative environmental impacts of lipid-rich wastewater is warranted [7].

Various physicochemical approaches including dissolved air flotation and chemical coagulation are employed for the treatment of lipid-rich wastewater [11-13]. However, these methods are encountered with drawbacks of high operational costs due to coagulation agents and generation of sludge with sedimentation problems [14]. Hence, the need for adoption of biological technologies involving application of bioproducts for an efficient, cost-effective, sustainable and eco-friendly treatment of high fat-containing wastewater [15]. The use of biosurfactants facilitate enzymatic activity and degradation of lipids by accelerating their solubility and bioavailability, thus eliminating the need for additional processes [16-18]. In addition, application of hydrolytic enzymes reduce the lipid and protein levels in the effluent via enzymatic catalysis and further enhance performance of the microbial population at a later phase of biological treatment [4,6,8,19-22].

In order to make bioemulsifiers and enzymes more favorable for use in biotechnological processes, different approaches, including immobilization have been employed for a cost-effective practice [23,24]. Insolubilization of bioproducts in a suitable support matrix offers many distinct advantages including resistance to environmental changes, easy recovery, multiple reuse, continuous operation, enhanced stability of the bioproducts under operational conditions and rapid termination of reactions [19,25-27].

Several studies on the combined use of free bioemulsifier and enzyme pool for oily wastewater treatment have been reported [14,17,28,29]. However, to the best of our knowledge, no studies have been reported on the comparative study of a mixture of free or immobilized bioemulsifier and hydrolytic enzymes (protease, lipase) for lipid-rich wastewater treatment. Therefore, this present study investigates the synergistic effects of free or immobilized extracellular glycoprotein bioemulsifier secreted from

Acinetobacter sp. Ab9-ES and hydrolytic enzymes (protease and lipase) produced from *Bacillus aryabhattai* in the reduction of pollutants from two different lipid-rich wastewater types.

2. Materials and methods

2.1. Materials

The bioproducts (hydrolytic enzymes and bioemulsifier) used in this experiment were produced by submerged fermentation from *Bacillus aryabhattai* and *Acinetobacter* sp. Ab9-ES, respectively. They exhibited robust activities at optimum pH and temperature of 8.0 and 50 °C (protease), 9.5 and 60 °C (lipase) and 7.0 and 50 °C (bioemulsifier). Sodium alginate used as support material for immobilization was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Immobilization protocol

The bioproducts were immobilized separately by entrapment method using the methods of Guleria et al. [30] and Ferhat et al. [31] with modifications. Bioproduct solution was added to a suitable amount of 2% (w/v) sodium alginate suspension. The mixture was extruded dropwise through a syringe into a $CaCl_2$ solution. When in contact with $CaCl_2$ solution, the alginate drops solidified and form beads, thus entrapping the bioproducts. The resultant beads were left to solidify for 1 h before washing with sterile distilled water for the removal of excess Ca^{2+} ions.

2.3. Collection of raw lipid-rich wastewater

Raw lipid-rich wastewater samples were collected from two different sources namely, dairy and poultry processing industries, all in the KwaZulu-Natal province of South Africa, into separate autoclaved 1 L Schott bottles. The wastewaters were generated mainly from production line, equipment, and floor cleaning operations.

2.4. Physicochemical characterization of lipid-rich wastewater

Raw lipid-rich wastewater samples were characterized for BOD, COD, TSS, total dissolved solid (TDS), turbidity, salinity, resistivity, electrical conductivity (EC), total nitrogen (TN), total phosphorus (TP), pH and temperature in accordance with standard methods [32]. Partition gravimetric method of Kirschman and Pomeroy [33] was used for the determination of lipid content while protein concentration was estimated according to the method of Lowry et al. [34] using bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as a standard. All analyses were performed in triplicate.

2.5. Batch biodegradation of lipid-rich wastewater

The synergistic effects of bioemulsifier and enzyme pool in the biodegradation of pollutants present in lipid-rich wastewater were investigated in 1 L Erlenmeyer flasks containing 500 ml sterile lipid-rich wastewater. The pH of the wastewater was adjusted to 8.0 using 1 N NaOH or 1 N HCl. Five percent (v/v) each of the free bioproducts or 2.5, 3 and 3.75 g of immobilized protease, lipase and bioemulsifier, respectively were added to the wastewater samples. This was followed by incubation under aerobic condition at 37 and 50 °C (150 rpm) for 120 h in a shaking incubator. A control flask without enzyme or bioemulsifier preparation was kept under similar conditions. Biodegradability of the lipid-rich wastewater samples was assessed by determining percentage removal efficiencies of COD and lipid contents at 24, 72 and 120 h using the formula shown in eq. (1) [35]. All analyses were conducted in triplicate.

$$\text{Removal efficiency (\%)} = \frac{\text{Ccontrol}-\text{Cf}}{\text{Ccontrol}} \times 100 \quad (1)$$

where $C_{control}$ is the value of COD or lipid content of the control lipid-rich wastewater, and C_f is the amount of these parameters after treatment.

2.6. Reusability potential of immobilized protease, lipase and bioemulsifier

Batch biodegradation of lipid-rich wastewater was repeated several times using a combination of immobilized protease, lipase and bioemulsifier. After each cycle, the beads were separated, washed with sterile distilled water and then added into a fresh wastewater sample. Percentage removal efficiencies of COD and lipid content were determined after every cycle. All experiments were carried out in triplicate.

3. Results and discussion

3.1. Characterization of lipid-rich wastewater

The physicochemical characteristics of dairy and poultry processing wastewaters were determined using standard procedures, and the mean values of the parameters are shown in Table 1. Both wastewater types are characterized with unpleasant odor coupled with whitish (dairy wastewater) and brownish (poultry processing wastewater) appearance. Furthermore, wastewater from poultry processing industry contained high concentrations of organic matter in form of lipids (88900 ± 10468 mg/L), COD (7518 ± 378 mg/L), BOD_5 (707 ± 13.58 mg/L), TDS (807000 ± 0.00 mg/L) and TSS (4667 ± 2887 mg/L) in comparison to dairy industry wastewater with lipid content (53367 ± 4306 mg/L), COD (5693 ± 17.56 mg/L), BOD_5 (691 ± 16.64 mg/L), TDS (5700 ± 0.00 mg/L) and TSS (2333 ± 1528 mg/L).

Table 1 Physicochemical characteristics of raw dairy and poultry processing wastewaters

Parameter	Dairy wastewater	Poultry processing wastewater
Color	Whitish	Brownish
Odor	Unpleasant	Unpleasant
Temperature (°C)	24.7 ± 1.53	22.6 ± 2.03
pH	12.55 ± 0.07	6.57 ± 0.10
COD (mg/L)	5693 ± 17.56	7518 ± 378
BOD_5 (mg/L)	691± 16.64	707 ± 13.58
TSS (mg/L)	2333 ± 1528	4667 ± 2887
TDS (mg/L)	5700 ± 0.00	807000 ± 0.00
Salinity (‰)	5.88 ± 0.00	0.81 ± 0.00
Resistivity (Ω.cm)	96 ± 0.00	620 ± 0.00
Total nitrogen (mg/L)	129 ± 3.21	79 ± 11.14
Total phosphorus (mg/L)	27.2 ± 1.31	24.3 ± 0.89
Lipid content (mg/L)	53367 ± 4306	88900 ± 10468
Turbidity (NTU)	1962 ± 7.21	2077 ± 83.37
Electrical conductivity (mS/cm)	10.42 ± 0.00	1614 ± 0.58
Protein content (mg/L)	785 ± 96	507 ± 40

*All values are expressed in mean; ± indicates SD

However, in both wastewater samples, the COD values were found to be higher than those of BOD, suggesting slow biodegradation of organic compounds in the wastewaters [36]. Similar characteristics of poultry and dairy wastewaters with high pollutant load have been reported in literatures [4,14,17,37-39]. In addition, pH of poultry processing wastewater was found to be acidic while that of dairy wastewater was extremely alkaline, probably due to availability of acidic and caustic cleaning agents, respectively in the wastewaters [13,40,41]. Furthermore, high salinity of dairy wastewater (5.88 ± 0.00‰) which is about 7-fold higher than that of poultry processing wastewater may be due to the presence of high concentrations of Na & Cl, resulting from the use of large amounts of alkaline cleaners in the dairy plant [42]. The electrical conductivity of poultry processing wastewater was 155-fold higher than that of dairy wastewater, an indication of the presence of high ionic substances in the wastewater [43]. The higher turbidity of poultry processing wastewater (2077 ± 83.37 NTU) in comparison to that of dairy wastewater may probably be due to the presence of residual blood, fats and intestinal content in the wastewater. The high nutrient level in dairy wastewater as compared to that of poultry processing wastewater is indicated by high TN (129 ± 3.21 mg/L) and TP (27.2 ± 1.31 mg/L) values, suggesting increased eutrophication risk of the receiving water bodies [13]. In general, variation in the physicochemical properties of the wastewaters to previously reported studies is a function of the type of products being produced and different operating procedures adopted at each plant [41]. The pollutant load of these wastewaters was found to exceed the South African guidelines for effluent discharge, thus necessitating treatment before disposal [44].

3.2. Simultaneous treatment of lipid-rich wastewater using a mixture of free or immobilized bioemulsifier and enzyme pool

The COD and lipid content represent crucial parameters for assessing the biodegradability of oily wastewater, as they give an idea about the amount of organic matter that was degraded during the treatment process [29,45]. In the present study, synergistic application of bioemulsifier and enzyme pool (protease and lipase) in the treatment of dairy and poultry processing wastewaters was investigated by carrying out a batch biodegradation experiment under different conditions. The rational for the choice of protease, lipase and bioemulsifier was due to high protein and lipid contents in the wastewater. High activity of bioproducts is a key prerequisite for industrial wastewater treatment [8]. Five percent (v/v) each of the bioproducts containing 151.14 U/ml, 81.43 U/ml and 83.3% of protease, lipase and emulsifying activities, respectively was used for the treatment of the wastewaters. Similar experiments were carried out concurrently using required amounts of immobilized protease, lipase and bioemulsifier with similar activities to the free bioproducts. In the first set of experiment, sterile wastewater samples without pH adjustment was incubated at 37 °C (150 rpm) for 120 h. Kinetics of degradation of pollutants in the wastewater was assessed by determining the reduction efficiencies of COD and lipid content at time intervals using standard methods. There was a significant reduction in the COD and lipid contents of the wastewater in comparison to control during treatment with free or immobilized bioproducts for 120 h. Maximum COD (60.51 & 65.19%) and lipid (47.98 & 63.53%) reduction efficiencies were recorded after 120 h in dairy wastewater when treated with the free and immobilized bioproducts, respectively (Fig. 1A & B). Maximum COD (86.44 & 93.65%) and lipid (51.62 & 69.06%) removal efficiencies found to be higher than those of dairy wastewater were obtained after 120 h treatment of poultry processing wastewater using free and entrapped bioproducts, respectively (Fig. 2A & B). The lower degradation of dairy wastewater compared to poultry processing wastewater suggests higher affinity of the bioproducts to poultry effluent constituents [3]. This may probably be due to the presence of high salt content inhibiting the degradation of pollutants in the dairy wastewater [46].

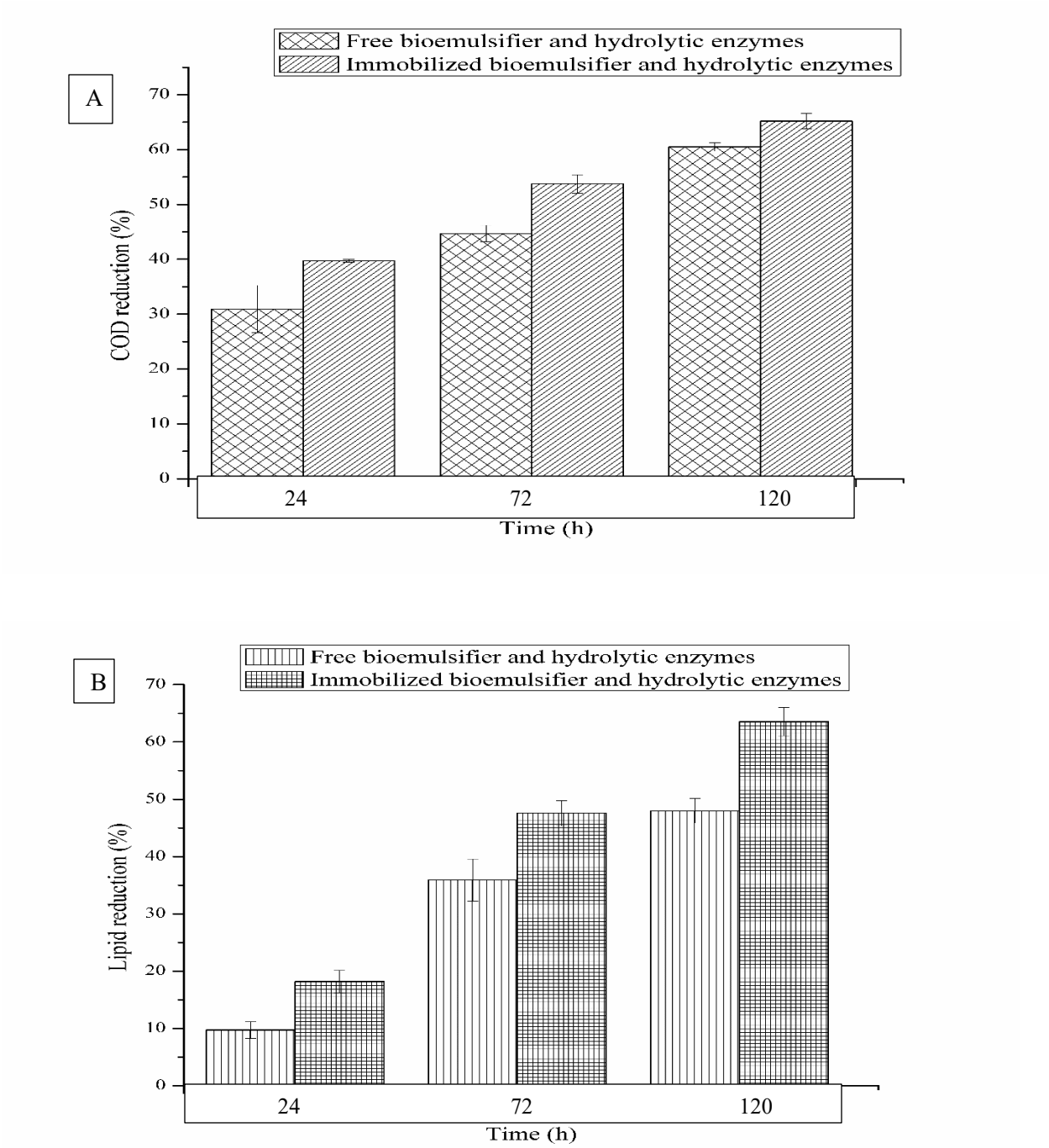

Fig. 1. Reduction in (A) COD and (B) lipid content during treatment of dairy wastewater using free or immobilized bioemulsifier and hydrolytic enzymes. The wastewater was treated at 37 °C without pH adjustment. Values indicate the average of triplicate values while the error bars represent the standard deviation.

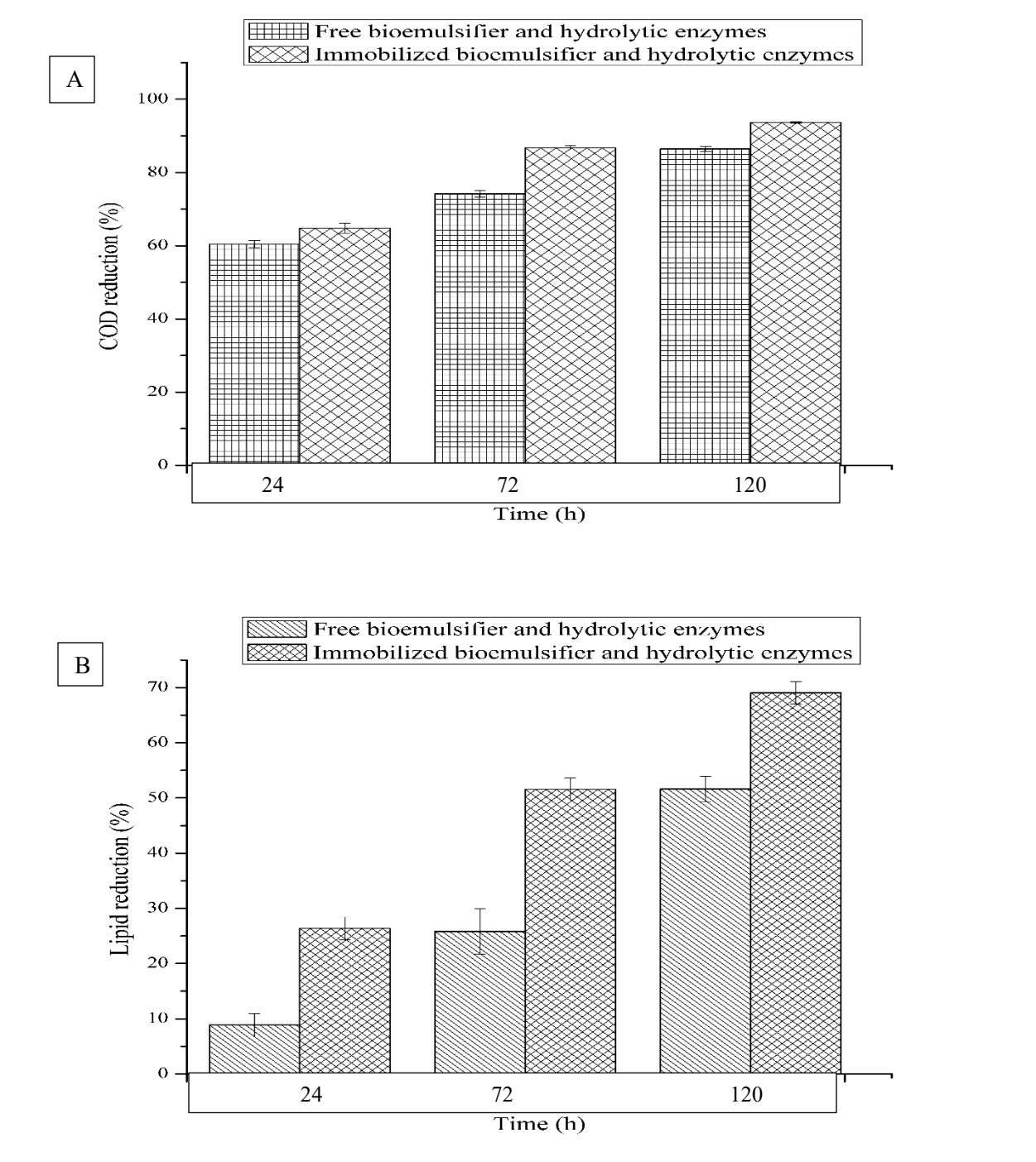

Fig. 2. Reduction in (A) COD and (B) lipid content during treatment of poultry processing wastewater using free or immobilized bioemulsifier and hydrolytic enzymes. The wastewater was treated at 37 °C without pH adjustment. Values indicate the average of triplicate values while the error bars represent the standard deviation.

In the second series of experiment, the pH of the wastewater samples was adjusted to 8.0 before addition of suitable amounts of free or immobilized bioproducts while the flasks were incubated at 50 °C for 120 h, since these bioproducts showed considerable activity at 50 °C and pH 8.0. During 120 h biodegradation of dairy wastewater, maximum reduction efficiencies of COD (65.96 & 77.52%) and lipid content (55.22 & 71.12%) were recorded at 72 h using free and immobilized bioproducts, respectively (Fig. 3A & B). In contrast to dairy wastewater, there was enhanced reduction of organic matter in poultry processing wastewater, with maximum COD (90.29 & 94.72%) and lipid (63 & 76.66%) removal efficiencies obtained at 72 h when treated with free and immobilized bioproducts, respectively (Fig. 4A & B). The observed decrease in the removal efficiencies of both wastewaters after 72 h incubation may be attributed to reduction in the activities of the bioproducts at longer treatment period, thus rendering them ineffective towards the bioconversion reactions [47]. This corroborates the findings of Jacobucci et al. [47] where a decrease in the degradation of oily wastewater was reported between 72-120 h during 5 d treatment with bioproduct produced from *Planococcus citreus*. In a similar study, Jeganathan et al. [45] investigated the hydrolysis of oil & grease (O & G) from pet food industrial wastewaters using immobilized lipase from *Candida rugosa*. Results obtained showed 65% and 69% reduction of COD and O & G, respectively during 72 h experiment. Dumore and Mukhopadhyay [48] reported reduction of 48% O & G and 47% COD from synthetic oily wastewater after 72 h treatment using chitosan-immobilized lipase from *Aspergillus niger*.

In general, the mixture of bioemulsifier and enzyme pool was found to be more effective in the reduction of COD and lipid from poultry processing wastewater in comparison to dairy wastewater. In addition, the immobilized bioproducts recorded higher pollutant removal efficiencies in contrast to the free counterparts, an indication of protective role provided by the support material, thus stabilizing the bioproducts and, as a consequence maintain their activities [25,49]. Such cocktail application of biosurfactant and enzyme pool has been reported for enhanced removal of COD and lipid from lipid-rich wastewater [14,17,29]. Furthermore, there was a slight improvement in pollutant degradation from wastewater treated at 50 °C and pH 8.0 in comparison to wastewater treated at 37 °C without pH adjustment. This may be due to the action of the bioproducts on the effluent organic matter, rendering little effect on the degradation of pollutants in the wastewater when treated at 50 °C and pH 8.0. Kanmani et al. [50] have reported similar results during batch enzymatic treatment of coconut mill effluent at 50 °C and pH 9.0. Results showed a moderate decrease in the removal efficiencies of COD (29%) and O & G (45%).

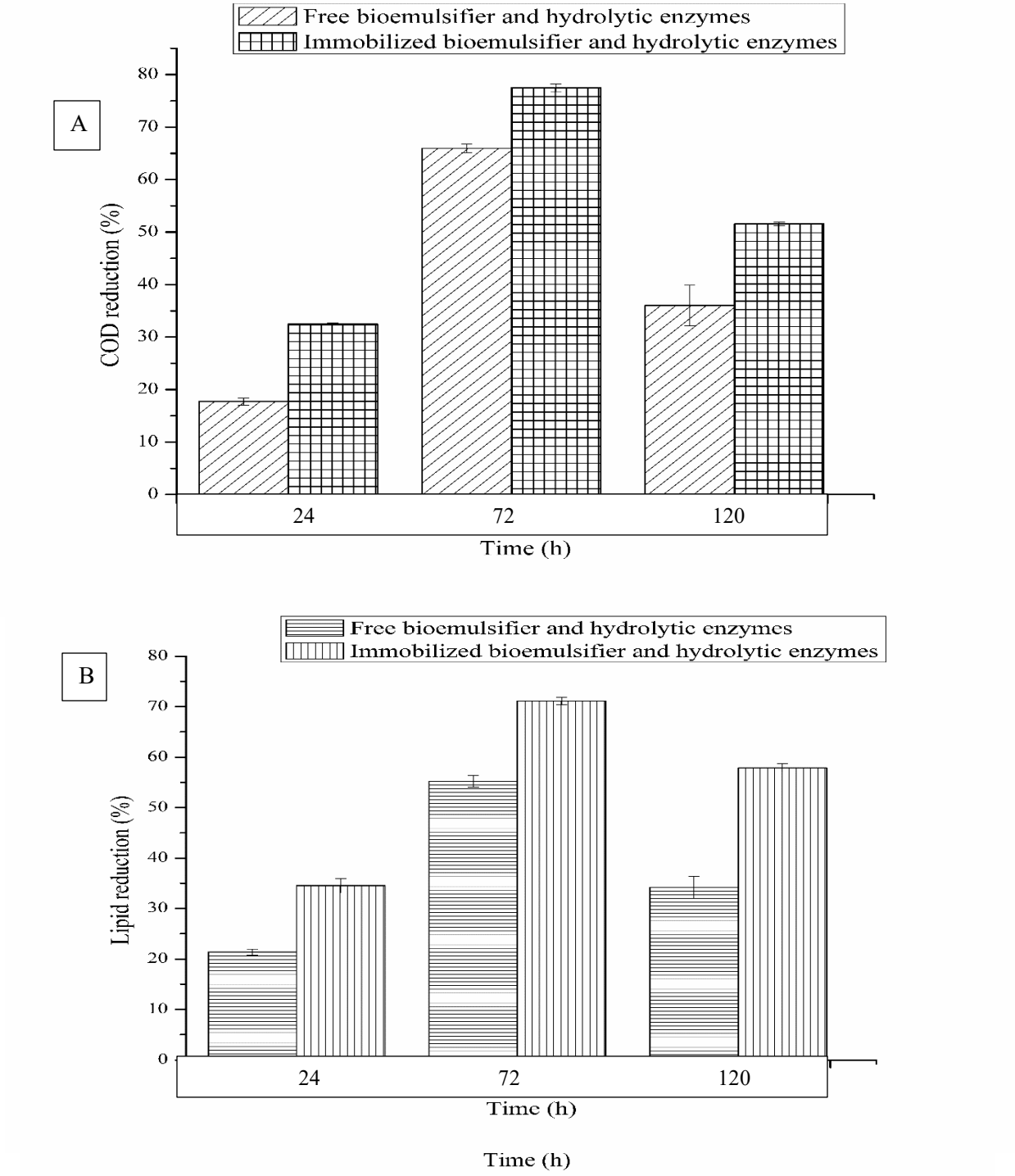

Fig. 3. Reduction in (A) COD and (B) lipid content during treatment of dairy wastewater using free or immobilized bioemulsifier and hydrolytic enzymes. The wastewater was treated at 50 °C and pH 8.0. Values indicate the average of triplicate values while the error bars represent the standard deviation.

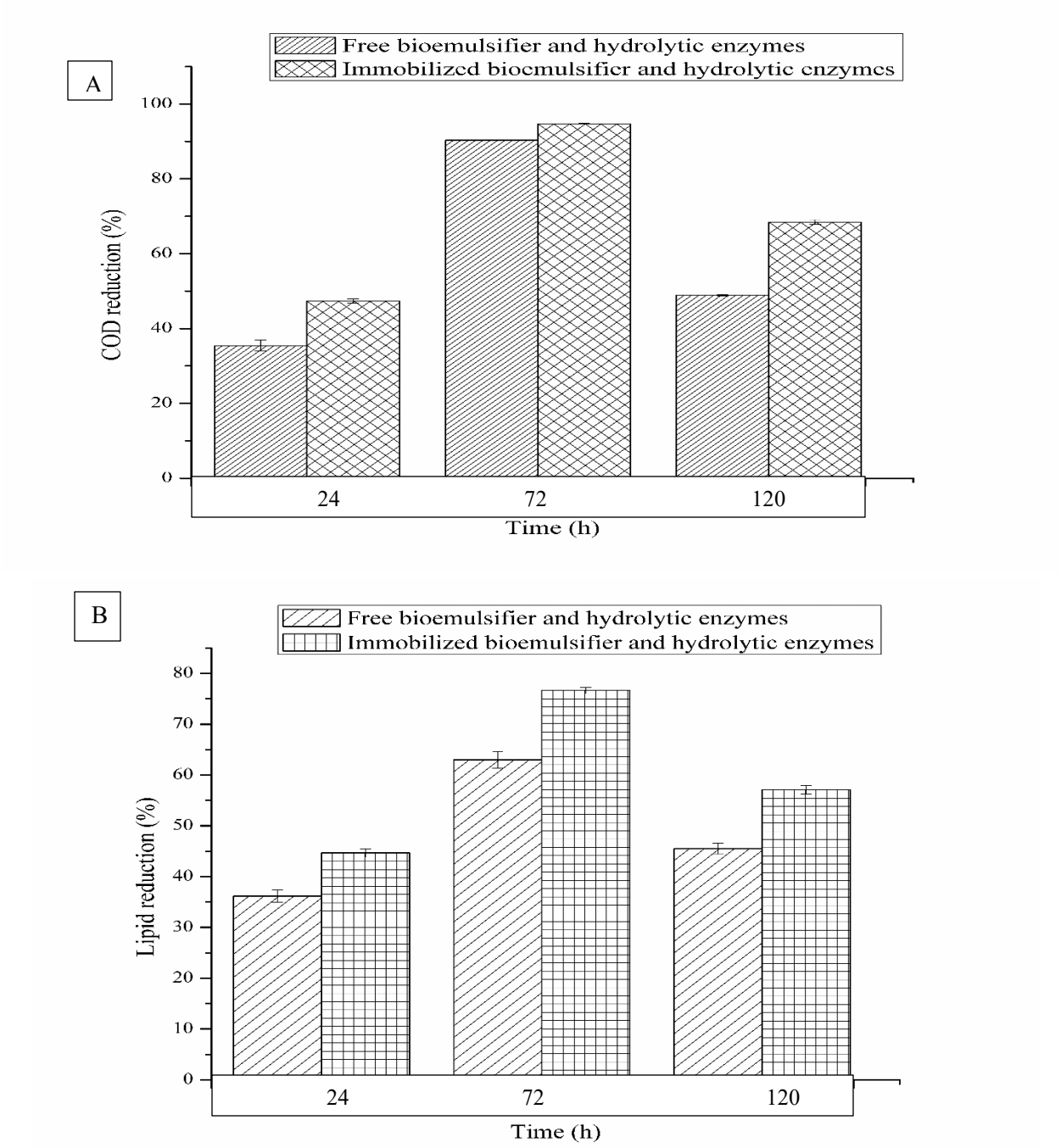

Fig. 4. Reduction in (A) COD and (B) lipid content during treatment of poultry processing wastewater using free or immobilized bioemulsifier and hydrolytic enzymes. The wastewater was treated at 50 °C and pH 8.0. Values indicate the average of triplicate values while the error bars represent the standard deviation.

3.3. Reusability potential of immobilized bioproducts

The reusability of immobilized bioemulsifier and enzymes is a key factor for a cost-effective application of the immobilizate for the degradation of pollutants in lipid-rich wastewater. Bioemulsifier and hydrolytic enzymes insolubilized in alginate gel beads were reused in several treatment batches of dairy and poultry processing wastewaters at 50 °C, pH 8.0 for 3 d, since considerable biodegradability was recorded under these optimum conditions. The cycle number affected the reduction of both COD and lipid content in the wastewater by the immobilized bioproducts. In dairy wastewater, the immobilized bioproducts could be reused for a maximum of six cycles for the reduction of COD and lipid content, retaining 22.18% and 17.94% removal efficiencies, respectively at sixth cycle (Fig. 5A). In the case of poultry processing wastewater, the immobilized bioproducts could be reused repeatedly for up to seven batches for the removal of COD and lipid, with reduction efficiencies of 40.07% and 30.44% recorded at the seventh cycle (Fig. 5B). The decreased degradation potential of the immobilized bioproducts in both wastewaters, after every cycle, is possibly due to leakage of the bioproducts from the beads or its inhibition by substrate/product molecules [45,50]. This supports the findings of Kanmani et al. [50], in which lipase immobilized in celite beads was repeatedly used for up to seven cycles during treatment of coconut mill effluent. Similarly, *Candida rugosa* lipase entrapped in alginate beads has been reported to be reused for up to four batches during enzymatic treatment of high strength lipid-rich wastewater [45].

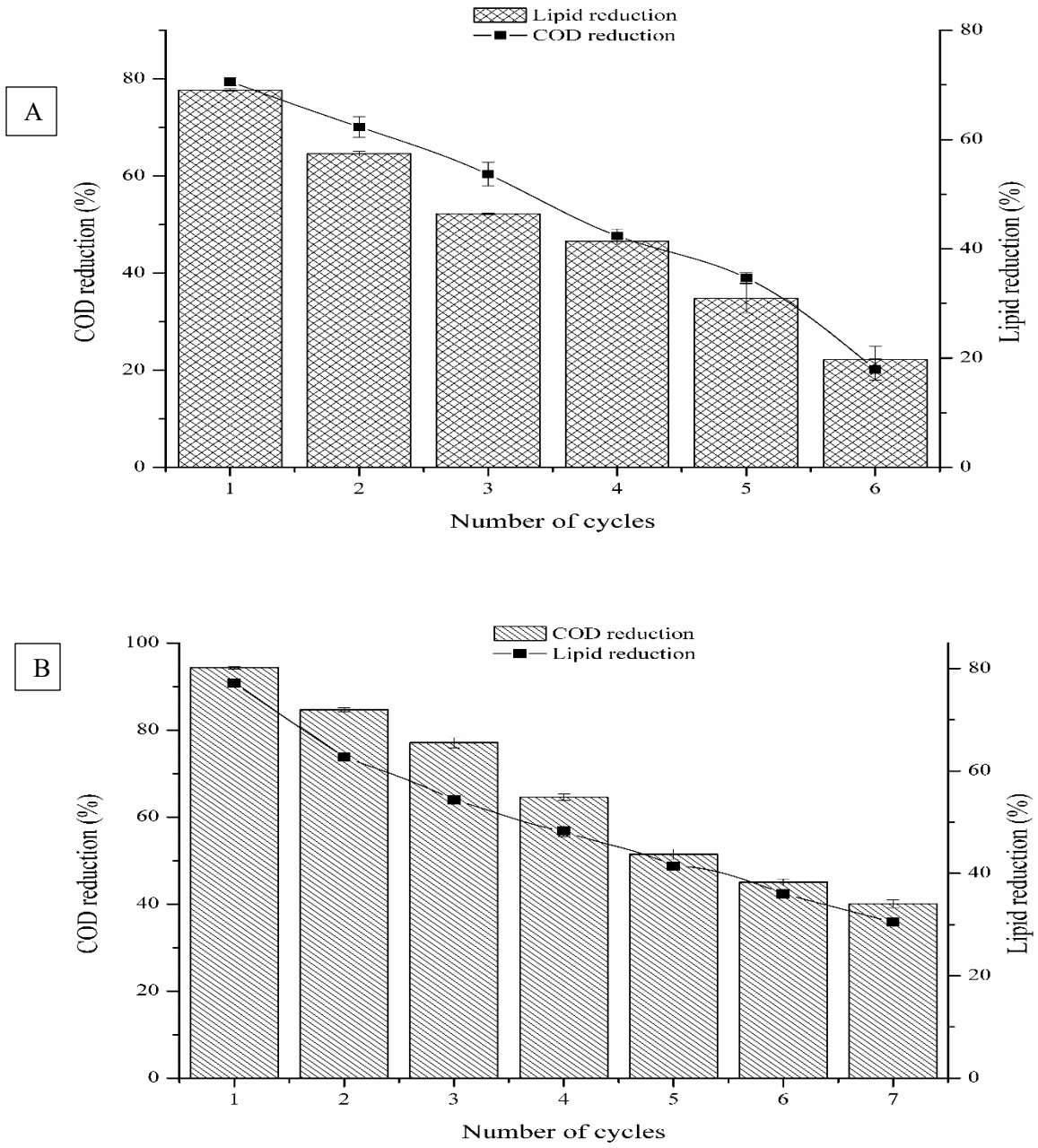

Fig. 5. Reusability potential of immobilized bioemulsifier and hydrolytic enzymes in the treatment of (A) dairy and (B) poultry processing wastewaters.

1. Conclusions

Mixture of free or immobilized bioemulsifier and hydrolytic enzymes was employed for the degradation of pollutants present in dairy and poultry processing wastewaters. Despite the high pollutant load of poultry processing wastewater, there was higher removal of COD and lipid from this wastewater at all the treatment conditions in comparison to dairy wastewater. In addition, the immobilized bioproducts were found to be more efficient in the degradation of organic load when compared to the free counterparts. Furthermore, the immobilized bioproducts could be reused for the treatment of dairy and poultry processing wastewaters.

The synergistic application of these bioproducts was found to be extremely advantageous in that, it is rapid, efficient and cost-effective in the reduction of pollutants present in lipid-rich wastewater in addition to eliminating the problem of generating solid waste. Thus, representing an attracting remedy to combat severe pollution problems emanating from the discharge of untreated and inadequately treated lipid-rich wastewater.

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CHAPTER ELEVEN

GENERAL DISCUSSION AND CONCLUSION

11.1 THE RESEARCH IN PERSPECTIVE

Dairy and poultry processing industries contribute tremendously to global industrial pollution. These industries consume large amounts of water for various processes, resulting in the discharge of huge volume of effluent containing high concentrations of organic molecules including fats and proteins, contributing to their low biodegradability coefficient (Liu and Haynes, 2011). Improper disposal of this wastewater, especially when untreated or inadequately treated poses serious threats to the receiving environment by causing air pollution and contamination of surface and underground water (Shete and Shinkar, 2013). Hence, the need for establishment of efficient, cost-effective, eco-friendly and sustainable technologies that can serve as alternatives to existing treatment systems for lipid-rich wastewater. Biological technology involving application of bioemulsifier and hydrolytic enzymes offers a promising approach for the effective treatment of high strength lipid-rich wastewater. These bioproducts facilitate degradation of lipid-rich wastewater by triggering two simultaneous processes namely; emulsification of fat particles and enzymatic catalysis, leading to increased reduction of organic load in the wastewater and alleviating the problem associated with the discharge of untreated wastewater (Damasceno *et al*. 2012, 2014; Parthipan *et al*., 2017). This project thus aimed at investigating the synergistic effects of a mixture of free or immobilized bioemulsifier and hydrolytic enzymes from indigenous bacterial isolates in the reduction of pollutants from dairy and poultry processing wastewaters.

Production and characterization of thermostable glycoprotein bioemulsifiers from indigenous *Acinetobacter* sp. isolated from lipid-rich wastewater in Durban was described in Chapter Five. High throughput techniques for screening and selection of bioemulsifier/biosurfactant-producing microorganisms are required to bioprospect for novel bioemulsifiers or bioemulsifier-producing microbial strains (Satpute *et al*., 2010). The use of single technique for the screening of bioemulsifier/biosurfactant-producing microorganisms is inaccurate and unreliable due to diverse functional and structural properties of the surface active compounds. As a result, *Acinetobacter* sp. Ab9-ES and Ab33-ES were screened for bioemulsifier/biosurfactant production by growing in the presence of different carbon sources including glucose, diesel or edible oils. The obtained culture supernatants were tested for surface/emulsifying activity using oil spreading test and emulsification assay. These study strains showed positive results for both tests, especially when tested with olive oil, since this edible oil was mostly utilized for growth and bioemulsifier/biosurfactant production by the bacterial strains. Surface tension (ST) was measured on the culture supernatants of *Acinetobacter* sp. in order to distinguish between bioemulsifier and biosurfactant

producers. The ST of the bacterial culture supernatants was found to be above the threshold of 40 mN/m, an indication that they are bioemulsifier producers (Monteiro *et al*., 2009; Franzetti *et al*., 2012; Lima *et al*., 2017; Patowary *et al*., 2017). Among microbial population, extracellular bioemulsifier production has been reported to be a common phenomenon among members of the genus *Acinetobacter* (Patil and Chopade, 2001; Jagtap *et al*., 2010). Growth of these organisms on water-insoluble carbon sources results in emulsification of the substrates in the culture medium, leading to the production of bioemulsifiers. Bioemulsifier production by these study strains was found to be growth-associated, with maximum emulsifying activities recorded during stationary (168 h) and exponential (120 h) growth phase for strains Ab9-ES and Ab33-ES, respectively. It is well established that bioemulsifier production is influenced by environmental factors, substrate composition and media concentration (Banat *et al*., 2014; Santos *et al*., 2016). Interaction of these parameters with each other in a multifaceted way affects the kinetics of biosurfactant production. Similar profile has been reported by several other bioemulsifier-producing microorganisms (Zheng *et al*., 2012; Gudiña *et al*., 2015; Heryani and Putra, 2017). The potential of these bioemulsifiers to emulsify different hydrophobic substrates was investigated in the presence of edible oils; aliphatic, aromatic and mixed hydrocarbons. The bioemulsifiers exhibited higher emulsification activity against edible oils, especially sunflower oil while less stable emulsions were formed with other hydrocarbons. The poor emulsification of the aliphatic, aromatic and mixed hydrocarbons may probably be due to the inability of the bioemulsifier to stabilize the microscopic droplets (Rufino *et al*., 2016). The emulsifying activity of the bioemulsifiers was substrate-dependent. Most microbial surface active compounds are substrate-specific and possess ability to emulsify different hydrocarbons at varying rates (Rufino *et al*., 2014; Bhattacharya *et al*., 2014). Findings from this study showed that the bioemulsifiers produced by *Acinetobacter* sp. possess higher emulsion-stabilizing capacity with different edible oils, therefore forming a potential biomolecule for the treatment of lipid-rich wastewater.

Effectiveness of bioemulsifier for various applications is dependent on their emulsifying activities over a broad range of temperature, pH and salinity (Markande *et al*. 2013; Rufino *et al*., 2016). These environmental factors affect the activity of bioemulsifiers in diverse ways. The emulsifier from *Acinetobacter* sp. in this study was subjected to varying conditions of pH, temperature, and salinity to determine the emulsifier's properties. The bioemulsifiers maintained stable emulsions over broad range of temperatures. However, slight decrease in emulsifying activity was observed at higher temperatures. This may be due to denaturation of protein component of the bioemulsifier during heat treatment (Sarrubo *et al*., 2006). Different bioemulsifiers with prolonged stability after exposure to higher temperatures have been reported (Shavandi *et al*., 2011). However, other surface active compounds lose some emulsifying activity at those temperatures (Vecino-Bello *et al*., 2012). The bioemulsifiers demonstrated emulsion-stabilizing

capacity over acidic to alkaline pH ranges, with maximum emulsifying activity recorded at neutral pH, found to be higher than that exhibited by synthetic surfactant. This corroborates the findings of Lima and Alegre (2009) and Aparna *et al*. (2012) in which bioemulsifiers produced by *Saccharomyces lipolytica* CCT-0913 and *Pseudomonas* sp. 2B, respectively were active at neutral pH. Similar profile has been observed at neutral pH in the stability of bioemulsifiers produced by *Rhocococcus* sp. TA6 and *Lactobacillus pentosus* (Shanvandi *et al*., 2011; Vecino-Bello *et al*., 2012). Bhattacharya *et al*. (2014) state that the decrease in emulsifying activity at extreme acidic pH, as reported in this study may be due to acid hydrolysis of carbohydrate moieties present in the biopolymer. Pronounced reduction in the emulsifying activity recorded at higher pH values may probably be due to some structural alterations of the bioemulsifiers under extreme pH conditions. In addition, emulsifying activity increases as the NaCl concentrations increase until attaining optimum at moderate concentration. Beyond these salinity levels, the emulsifying activity declined drastically. The salinity had significant effect on hydrocarbon emulsification and stability due to ionization caused by the interaction of the salt ions with the hydrophobic substrates (Amodu *et al*., 2014). In general, the robust characteristics of these bioemulsifiers are very beneficial for applications under extreme conditions of temperature, moderate pH and salinity in bioremediation of oil-contaminated sites.

Chapter Six investigates the statistical optimization of bioprocess factors for enhanced production of protease from *Bacillus aryabhattai* Ab15-ES using response surface methodology (RSM). *Bacillus aryabhattai* Ab15-ES isolated from lipid-rich wastewater was screened for proteolytic activity using skim milk agar. The formation of clear zone around the agar wells, resulting from hydrolysis of casein was an indication of protease production (Olajuyigbe and Ajele, 2005). Such high extracellular enzymatic activity could largely be due to high rates of substrate utilization and hydrolysis by the bacterial isolate. Extracellular protease production by microbial strains is constitutive or partially inducible in nature. As a result, the type of substrate utilized in the fermentation medium mostly influences the enzyme synthesis. Therefore, selection of appropriate inducible substrate and microorganisms is paramount for the production of desired metabolite (Bhunia *et al*., 2012). Preliminary studies of protease production by *Bacillus aryabhattai* Ab15-ES yielded maximum enzyme production during late exponential phase of growth, and this was found to be growth-linked. Optimum protease production from *Bacillus licheniformis* N-2 (Nadeem *et al*., 2008) and *Bacillus subtilis* SHS-04 (Olajuyigbe, 2013) during late exponential phase has been reported. The rapid decline in protease production beyond optimum may be due to depletion of nutrients in the growth media, or autolysis resulting from build-up of the enzyme in the production media (Nadeem *et al*., 2008). The formation and accumulation of extracellular enzymes in the fermentation media depend on media constituents such as carbon and nitrogen sources, which are vital parameters influencing

enzyme production (Akolkar *et al*., 2009). As a result, conventional optimization studies involving change of 'one-variable-at-a-time' was used for the optimization of process parameters to achieve optimum cell density and enhanced production of microbial metabolite. Of the carbon sources tested, maltose was found to result in maximum protease production by *Bacillus aryabhattai* Ab15-ES. This supports the findings of Raj *et al*. (2012) and Vijayaraghavan *et al*. (2014) where highest protease yields were attained in a medium containing maltose as a carbon source. The optimum protease synthesis recorded in the presence of beef extract may be due to the presence of multi-nutrient elements including free amino acids, essential fatty acids, carbohydrates, etc. in the organic nitrogen sources, which induced the growth of the organism for maximum protease production (Esakkiraj *et al*., 2011). Several authors have recorded similar results (Umuyaparvathi *et al*., 2013; Kumar *et al*., 2014). However, these approaches are time-consuming and thus expensive for simultaneous consideration of many variables. In addition, they are laborious, require more experimental data and unable to detect interactions and optimal conditions among variables (Li *et al*., 2008). Hence, the need for statistical optimization of bioprocess parameters for maximum protease production (Singh and Bajaj, 2016). Statistical experimental method is widely used in biotechnology to maintain a balance between various parameters for enhanced protease production (Chandra *et al*., 2015). The central composite design (CCD) of RSM used in the present study was very useful in confirming the effects and interactions of fermentation variables. In addition, it results in enhanced productivity, reduces process changeability, and gives closer confirmation of the predicted response to the experimental values. Enhancement of protease production from *Bacillus aryabhattai* Ab15-ES by RSM was achieved under the optimum conditions of temperature, pH, inoculum volume, maltose and beef extract, resulting in a 4.4-fold enhancement in protease production using the basal medium as reference. This was found to be higher than that of *Bacillus* sp. HTS102 (Queiroga *et al*., 2012) and *Brevibacterium linens* DSM 20158 (Shabbiri *et al*., 2012). These results suggest that the fermentation parameters greatly influence extracellular protease production by the bacterial strain and their interactions are crucial in the synthesis of the enzyme (Rao *et al*., 2008).

Bacillus aryabhattai SE3-PB was isolated from lipid-rich wastewater, and found to exhibit lipolytic activity following screening on Tween-20 and phenol red agar (Gopinath *et al*., 2006; Singh *et al*., 2006). Lipase production from *Bacillus aryabhattai* SE3-PB by submerged fermentation started at early log phase and reached optimum during late exponential growth phase. The decrease in lipase production recorded beyond exponential phase may probably be due to the presence of inhibitors (e.g. protease) or limitation of nutrients in the culture medium. The supplementation of fermentation medium with inducible substrates enhances lipase production, since the carbon chain moiety of the fatty acids present in triacylglycerol controls lipase production (Sethi *et al*., 2016). In the present study, olive oil in the fermentation medium

for preliminary lipase production was replaced with commercial vegetable oils viz. palm oil, castor oil, rice bran oil, sunflower oil or canola oil in order to determine best oil that will induce maximum extracellular lipase production by *Bacillus aryabhattai* SE3-PB. Lipase production varies in the presence of the different inducer oils, with maximum lipase production recorded in the fermentation media containing sunflower oil as inducer (Chapter Seven). In a similar study, Facchini *et al*. (2015) and Golani *et al*. (2016) recorded maximum lipase production by *Fusarium verticillioides* and *Staphylococcus chromogenes* 01 A, respectively in the presence of sunflower oil as an inducible substrate. Face centered CCD of RSM used in the present study for optimization of fermentation conditions including temperature, pH, inoculum volume, agitation and sunflower oil concentration for improved lipase production from *Bacillus aryabhattai* SE3-PB indicated the importance of these parameters at different levels, and their interactions. Lipase production was significantly influenced by temperature, pH, inoculum volume, agitation and sunflower oil concentration. A high similarity recorded between the predicted and observed results suggests that the model was accurate for process optimization, resulting in 7.2-fold enhancement in lipase production when using unoptimized medium as reference. Improvement of lipase production by *Bacillus aryabhattai* SE3-PB was largely higher than that of *Burkholderia* sp. (Lo *et al*., 2012) and *Acinetobacte*r sp. AU07 (Gururaj *et al*., 2016). The present study suggests a rational choice of optimum processing conditions for large scale and cost-effective production of lipase by *Bacillus aryabhattai* SE3-PB for various biotechnological applications.

Biochemical and kinetics properties of free and immobilized protease and lipase from *Bacillus aryabhattai* were investigated in this study and reported in chapter Eight and Nine. The partially purified enzymes were immobilized in alginate beads by entrapment method. Enzyme immobilization was greatly influenced by varying sodium alginate and calcium chloride concentrations. Comparative study of biochemical profiling of both free and immobilized enzymes revealed no change in the optimum temperature and pH of both enzyme preparations for maximum activity. This suggested that the chemical coupling of the enzymes to the polymer had no effects on temperature or pH profile. Ibrahim *et al*. (2016) and Xiao *et al*. (2016) have also observed similar phenomenon in which no change was recorded in the optimum temperature and pH of biocatalysts before and after immobilization. However, in comparison to free enzymes, the immobilized enzymes recorded enhanced stability over the investigated pH and temperature ranges. Ranjbakhsh *et al*. (2012) and Cao *et al*. (2016) observed that the significant improvement in the stability of the entrapped enzyme might be due to immobilization in the support material, which protect the tertiary structure of the enzyme and prevent conformational transition of the enzyme. Kinetics properties of the enzymes revealed enhanced enzyme-substrate affinity and increased catalytic efficiency of the immobilized enzymes when compared to soluble enzymes. In addition, the immobilized enzymes were more stable when stored at 4

and 25 °C for 30 d and reusable at several consecutive cycles, indicating a good operational stability of the biocatalysts (Srivastava *et al*., 2014). This was not surprising, since soluble enzymes are not stable under prolonged storage thus, leading to a reduction in catalytic activity (Çevik *et al*., 2011). Reusability of insolubilized enzymes is recognized as one of the most vital factors that influence their cost-effective application for various bioprocesses (Ibrahim *et al*., 2016). Findings from this study suggest the efficient and sustainable use of the developed immobilized catalytic system for various environmental and biotechnological applications.

The present study investigates the synergistic effects of a mixture of free or immobilized extracellular glycoprotein bioemulsifier and hydrolytic enzymes (protease and lipase) in the reduction of organic load from dairy and poultry processing wastewaters. Kinetics study of degradation of organic load in the wastewater revealed maximum chemical oxygen demand (COD) and lipid removal efficiencies in dairy and poultry processing wastewaters when treated with free or immobilized bioproducts (Chapter Ten). However, in comparison to dairy wastewater, there was enhanced reduction of organic load from poultry processing wastewater, suggesting higher affinity of the bioproducts to poultry effluent constituents (Valladão *et al*., 2011). The immobilized bioproducts were found to be more effective in the degradation of pollutants from both wastewater types when compared to the free counterpart, an indication of protective role provided by the support material in stabilizing the bioproducts thus, maintaining their activities (Homaei *et al*., 2013). Such cocktail application of biosurfactant and enzyme pool has been reported for enhanced removal of COD and lipid from lipid-rich wastewater (Damasceno *et al*., 2012, 2014). In addition, the immobilized bioproducts were reusable consecutively for the reduction of pollutants in the wastewater. The decreased degradation potential of the immobilized bioproducts in both wastewater types, after every cycle, is possibly due to leakage of the bioproducts from the beads or its inhibition by substrate/product molecules (Jeganathan *et al*., 2006). This supports the findings of Kanmani *et al*. (2015), in which lipase immobilized in celite beads was repeatedly used for up to seven cycles during treatment of coconut mill effluent. The synergistic application of these bioproducts was found to be extremely advantageous in that, it is rapid, efficient and cost-effective in the reduction of pollutants present in lipid-rich wastewater in addition to eliminating the problem of generating solid waste. Thus, representing an attractive remedy to combat severe pollution problems emanating from the discharge of untreated and inadequately treated lipid-rich wastewater.

11.2 POTENTIAL FOR FUTURE DEVELOPMENT OF THE STUDY

Biological method is an emerging treatment technology that utilizes metabolic potential of microorganisms for the elimination of hazardous pollutants present in lipid-rich wastewater under aerobic or anaerobic

conditions, or a combination of both *via* complete degradation or sequestration (Soleimaninanadegani and Manshad, 2014; Nzila *et al*., 2017). In the present study, the potential of a mixture of free or immobilized bioemulsifier and hydrolytic enzymes (protease and lipase) for the reduction of pollutants present in dairy and poultry processing wastewaters was investigated under aerobic conditions. Anaerobic degradation of these wastewater types using these bioproducts could be a promising strategy for large-scale production of biogas (methane), thus serving as a cheap and alternative source of energy. This energy can be used to power a biological wastewater treatment plant making them self-sustainable. This will help to lower the reliance on non-renewable energy sources such as fossil fuels, thus reducing carbon footprint and expenditure on energy production. In addition, utilization of agro-industrial residues and industrial effluents can serve as a cheaper substrate for a cost-effective production of bioemulsifier and hydrolytic enzymes. Cloning and expression of bioproduct (bioemulsifier, protease, lipase) genes from *Acinetobacter* sp. and *Bacillus aryabhattai* in suitable prokaryotic hosts could be a rapid technique for large scale production of these metabolites. Furthermore, these bioproducts could be applied for the treatment of wastewater with various organic load in order to ascertain the robustness of the system. Optimization of process parameters using statistical experimental designs for the treatment of lipid-rich wastewater could help in the selection of significant factor, study interaction among the parameters and search for optimum conditions for maximum degradation of pollutants in the dairy and processing wastewaters. Future studies may also focus on proper understanding of the behavior of pollutants in the lipid-rich wastewater and mechanisms of their degradation through combined use of bioemulsifier and hydrolytic enzymes.

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APPENDIX I

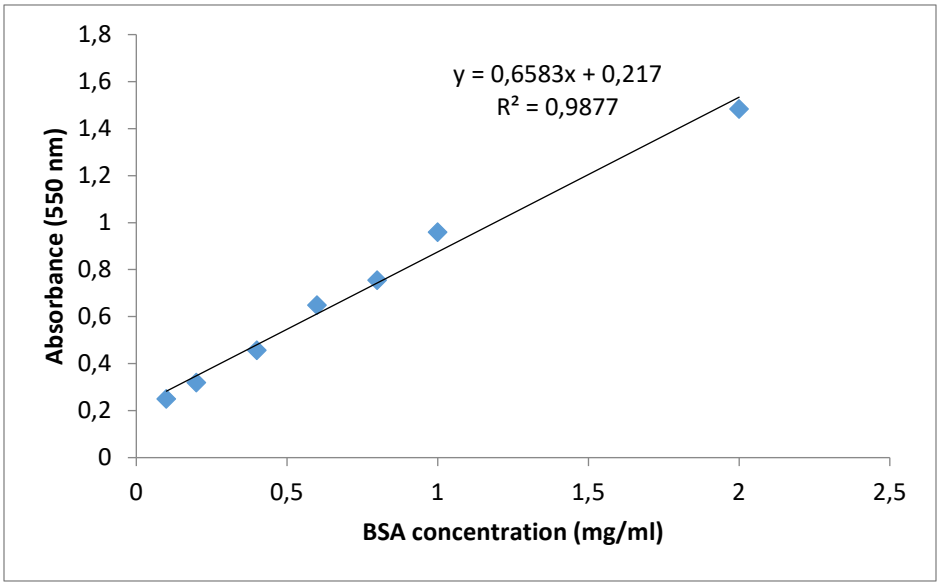

Fig. 1: Standard curve for estimation of total protein

APPENDIX II

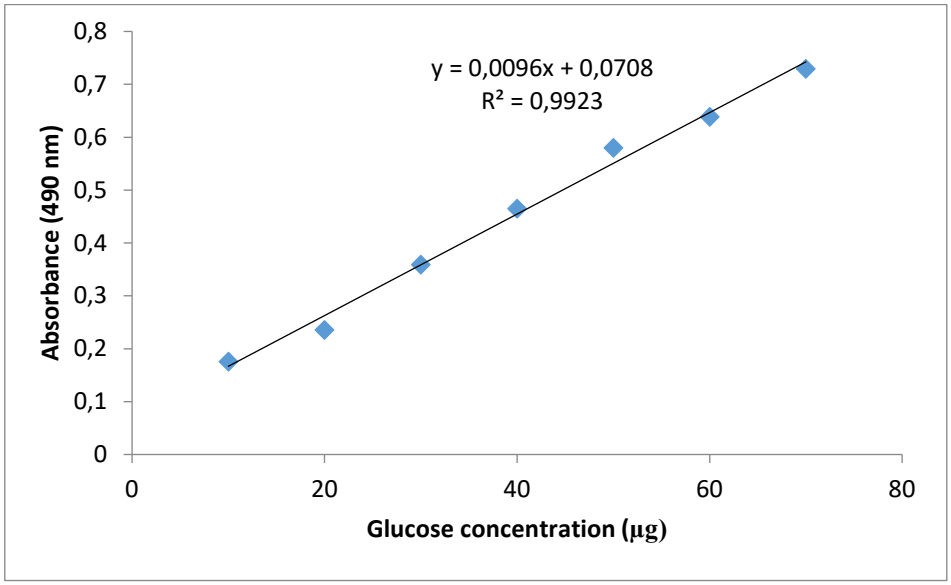

Fig. 2: Standard curve for determination of carbohydrate

APPENDIX III

Table 1: Replicate values for the physicochemical characterization of dairy and poultry processing wastewaters

Parameter	Dairy wastewater	Poultry processing wastewater
Temperature (ºC)	25	21
	26	24.9
	23	22
pH	12.61	6.60
	12.48	6.65
	12.57	6.46
COD (mg/L)	5695	7185
	5710	7930
	5675	7440
BOD_5 (mg/L)	672	691
	703	714
	698	715
TSS (mg/L)	1000	3000
	2000	3000
	4000	8000
TDS (mg/L)	5.70	807
	5.70	807
	5.70	807
Salinity (‰)	5.88	0.81
	5.88	0.81
	5.88	0.81
Resistivity (Ω.cm)	96	620
	96	620
	96	620
Total nitrogen (mg/L)	131	91
	130	69
	125	77
Total phosphorus (mg/L)	27.4	24.6
	25.8	25
	28.4	23.3
Lipid content (mg/L)	56099	98436
	55600	77700
	48403	90565
Turbidity (NTU)	1968	2172
	1964	2016
	1954	2043
Electrical conductivity (mS/cm)	10.42	1614
	10.42	1614
	10.42	1614
Protein content (mg/L)	682	537
	871	463
	803	521