Synthesis, Characterization and the Antimicrobial effect of diiodothiocyanate (I_2SCN^-) on *Escherichia coli* and *Enterococcus faecalis*

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Synthesis, Characterization and the Antimicrobial effect of diiodothiocyanate (I_2SCN^-) on *Escherichia coli* and *Enterococcus faecalis*

A dissertation submitted to the University of KwaZulu-Natal fulfilling the academic requirements for the Degree of Master of Science

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Declaration

I hereby declare that the experimental work and results presented in this thesis are the original work of my study carried out in the School of Chemistry and Physics, University of KwaZulu-Natal, Pietermaritzburg campus, and has never been submitted for the fulfillment of any degree at any institution.

[redacted]

Nomaswazi Madonsela (Student)

I hereby certify that this is correct.

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Dedications

I dedicate this work to God Almighty, my creator, my pillar of strength, and my source of hope. I am grateful to Nomthandazo Madonsela and my entire family, for their unconditional love and support.

Abstract

The threat posed by antimicrobial resistance is rising to dangerously high levels worldwide. However, chemical species such as iodide-based interpseudohalogen are attracting attention as potential new class of antimicrobials. Previously, these compounds were ignored based on low availability of I^- in most physiological fluids. Recently, it was shown that the peroxidase system LPO-H_2O_2-SCN^-/I^- produces a highly cytotoxic cyanogen iodide (ICN) which plays a key role in killing bacteria. Research efforts to find new chemical species with antimicrobial properties from non-enzymic reactions of iodine (I_2/I^-) with thiocyanate (SCN^-) are currently underway. Herein, we describe the formation of the interpseudohalogen, diiodothiocyanate (I_2SCN^-) in two different media, i.e. water and acetonitrile. The formation of I_2SCN^- was confirmed by ^{13}C NMR spectroscopy, UV-visible spectrophotometer, stopped-flow technique and Job's plot. The biological activities of I_2SCN^- against strains of *Escherichia coli* and *Enterococcus faecalis* bacteria were determined by zone of inhibition plate tests and traditional optical density (OD) measurements. Reaction mixtures of I_2 and SCN^- at various ratios showed inhibition against both Gram-positive (*E faecalis)* and Gram-negative (*E coli*) strains of bacteria. The results indicate that I_2SCN^- was more effective against *E coli* compared to *E faecalis* due to differences in the structural features of the two bacteria. The *E faecalis* has a thicker cell wall compared to *E coli* which makes it difficult for any antibacterial compound to penetrate and kill the bacteria. Reaction mixtures of I_2 and SCN^- at various ratios showed antibacterial activity against both Gram-positive (*E. faecalis)* and Gram-negative (*E. coli*) strains of bacteria, with the equimolar solution being the most efficient. The I_2SCN^- compound was more effective against *E. coli* compared to *E. faecalis.* This is due to differences in the structural features of the two bacteria, *E. faecalis* has a thicker cell wall compared to *E. coli* which makes it difficult for antibacterial compounds to penetrate the bacteria.

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List of Abbreviations

A	Absorbance
ATCC	American Type Culture Collection
AST	Antibiotic susceptibility testing
AMR	Antimicrobial resistance
CH_3CN	Acetonitrile
BHI	Brain Heart Infusion
Br^-	Bromine ions
BHI	Brain Heart Infusion
Cl^-	Chlorine ions
Cys	Cysteine
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
I_2SCN^-	Diiodothiocyanate
$I(SCN)_2^-$	Dithiocyanate iodide
E. faecalis	*Enterococcus faecalis*
E. Coli	*Escherichia Coli*
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
$OH\cdot$	Hydroxyl radical
HOBr	Hypobromous acid
HOCl	Hypochlorous acid
HOI	Hypoiodous acid
HOSCN	Hypothiocyanite
I_2	Iodine
I^-	Iodine ions
LPO	Lactoperoxidase
λmax	Maximum absorbance
Min	Minute
MDR	Multidrug resistant
MPO	Myeloperoxidase

NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric Oxide
NA	Nutrient agar
NB	Nutrient Broth
OD	Optical density
ROS	Reactive oxygen species
SPO	Salivary peroxidase
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
Na_2HPO_4	Sodium phosphate dibasic
$NaH_2PO_4 \cdot 2H_2O$	Sodium phosphate monobasic dehydrate
NaSCN	Sodium thiocyanate
SOD	Superoxide dismutase
$O_2^{\cdot}$	Superoxide radical
RNA	Ribonucleic acid
SCN^-	Thiocyanate ions
I_3	Triidide
ZOI	Zone of inhibition

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Chapter 1: Introduction

1.1 Antimicrobial Resistance

The overuse and misuse of antibiotics have contributed significantly to bacterial resistance.[1] To date, antimicrobial resistance has emerged towards most novel antibiotics that have been developed. The spread of antimicrobial resistance is a global public health challenge, as increasing numbers of deaths reported annually are attributed to it.[2] The leading causes of antimicrobial resistance are well-known and include overuse of antibiotics in animal farming, over-prescription of antibiotics, and poor hygiene.[1] The alarming rate of spreading antimicrobial resistance has initiated global efforts to develop new antimicrobial alternatives. The fight against antimicrobial resistance involves understanding bacteria's genetic makeup and developing new antimicrobial agents to support the existing immune system.

ESKAPE pathogens are a group of representative bacteria, namely, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa, and Escherichia coli*.[3-7] These bacteria have through mechanisms involving genetic mutation developed resistance against existing antimicrobials therapies. Five years ago, the world health organization (WHO) identified ESKAPE bacteria for investigation in order to develop new and more effective antimicrobial species.[8] In the field of antimicrobial resistance, it is known that ESKAPE pathogens are the leading cause of hospital infections worldwide.[9, 10] Understanding the resistance mechanisms of these bacteria is necessary for the development of novel antimicrobial agents. Some of the antimicrobial resistance mechanisms used by the ESKAPE pathogens include enzyme inactivation, modification of their target sites and changing cell permeability.[4] Since the discovery of penicillin about 90 years ago, the efficacy of many commonly used antibiotics has continued to lessen.[11] Research effort is now focussed on the discovery of new antibiotics derived from microbial sources, or on the synthesis of new compounds.

Antibiotics are highly cytotoxic towards bacteria and function in multiple pathways to weaken the bacterial cell wall or membrane. It is a process that activates due to physical interaction between the drug molecule and its specific target in bacteria. This process is followed by the affected bacterium's biochemical, molecular, and ultrastructural alterations.[12] They induce bacterial cell death mainly by inhibiting protein (the ribosome and associated proteins),

deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) synthesis, Figure 1.1.[12-14] Alternatively, antibiotics can also enter bacteria's cell walls by binding to them and transporting them through energy-dependent transport mechanisms in ribosomal sites, which inhibits protein synthesis.[13, 15]

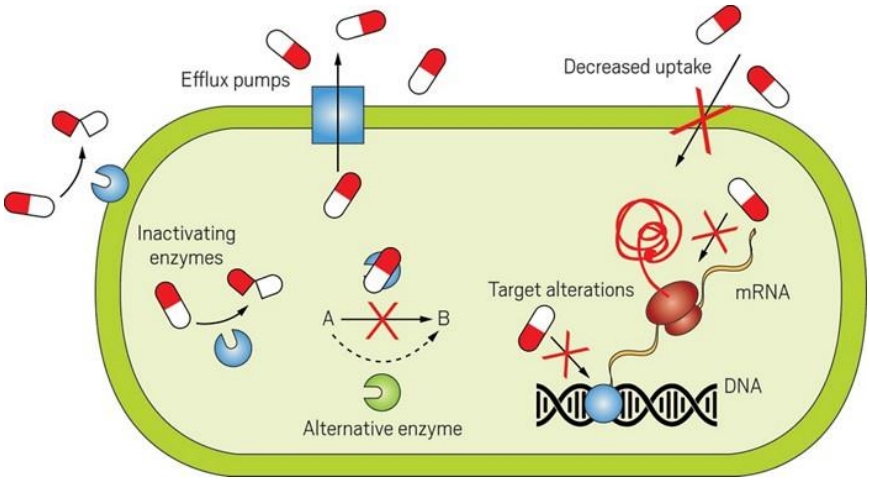

Figure 1.1 Antibiotic resistance in bacteria.[14]

1.2 Human Defensive System

Comprehensive efforts to develop new antibiotic alternatives to curb antimicrobial resistance are ongoing, with researchers studying resistance mechanisms, innate peroxidase systems, and antimicrobial agents.[16, 17] Phagocytic cells such as neutrophils are first responders of host defence to be recruited to the area of bacterial invasion and myeloperoxidase (MPO) is the critical component in its antimicrobial defence .[18] Innate host defence systems of exocrine fluids such as saliva and milk are peroxidase based and employ hydrogen peroxide (H_2O_2) as an oxidant of halides and pseudohalides to produce antimicrobials that are more effective than H_2O_2.

Neutrophils are specialized cells that hunt, engulf, and kill invading microorganisms. The circulating neutrophils are short-lived and are estimated to survive for between eight to twelve hours in the blood and one to two days in tissues.[19] When triggered, neutrophils initiate the killing by rapidly increasing the release of reactive oxygen species (superoxide and hydrogen peroxide), a process known as respiratory burst. Activated neutrophils use nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Figure 1.2) to catalyse the

reduction of molecular oxygen to superoxide radical ($O_2^{\bullet -}$). The chemically unreactive superoxide is dismutated to hydrogen peroxide (H_2O_2), a process catalyzed by superoxide dismutase (SOD) in mammalian cells.

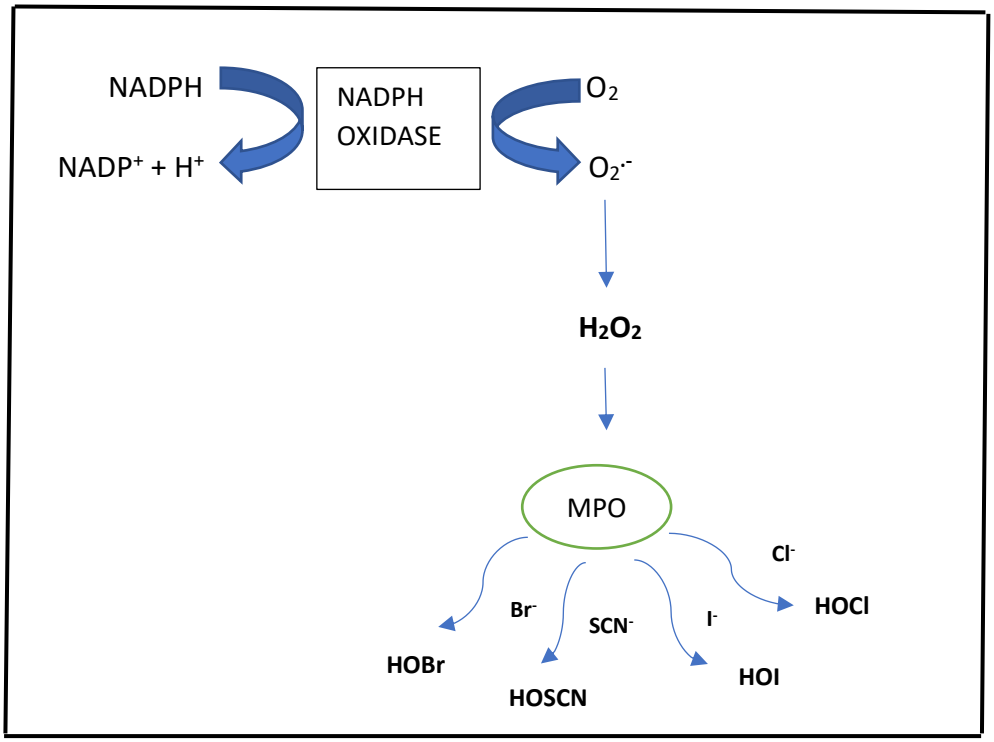

Figure 1.2: Possible oxidants generated in Myeloperoxidase system.

There are two defensive peroxidase systems in the oral cavity: salivary peroxidase (SPO) and myeloperoxidase (MPO). It has been estimated that approximately 75% of the peroxidase activity in saliva is due to MPO, with the remaining activity attributed to SPO.[20] Other defensive peroxidase systems especially those found in breast milk and mucosal lining of lungs use lactoperoxidase (LPO). SPO is structurally and catalytically similar to LPO.[21] The peroxidase system consists of an enzyme, the substrate, hydrogen peroxide and a halogen (Cl^-, Br^- and I^-) or a pseudohalogen (SCN^-). *In vivo*, the SPO and LPO systems prefer to only employ the pseudohalide SCN^- as a substrate to produce $OSCN^-$. SPO and LPO are coded for the same gene.[22] In contrast to peroxidases that essentially only employs SCN^- as a substrate (e.g. LPO and SPO), the MPO system is capable of oxidizing Cl^- to produce hypochlorite (OCl^-). Similarly, hypobromite (OBr^-) can also be generated by the MPO system, but only in minor amounts in the oral cavity.[23] The catalytic mechanism of MPO is given by equations 1 – 3.

$$MPO + H_2O_2 \rightleftharpoons MPO\text{-}I \qquad (\mathbf{1})$$

$$MPO\text{-}I + X^- \rightleftharpoons MPO + OX^- \qquad (\mathbf{2})$$

$$OX^- + H^+ \rightleftharpoons HOX \qquad (\mathbf{3})$$

Iodide (I^-) is a kinetically favoured substrate of all human defence peroxidases and is recognised to produce more effective iodine-derived antimicrobials than any of the other known products of peroxidases. The peroxidase/H_2O_2/I^- system oxidizes I^- to I^+ results in a mixture of species such as I_2/I_3^-/I_2OH/HOI depending on the pH and I^- concentration of the system.[24, 25]

For a long time, the reaction between I_2 and SCN^- in aqueous solution under normal conditions has been known in the chemical literature[26, 27], and it is characterized by the generation of inter-pseudo-halogen cyanogen iodide:

$$4I_2 + SCN^- + 4H_2O \longrightarrow 7I^- + 8H^+ + ICN + SO_4^{2-} \qquad (\mathbf{4})$$

Oxidation of iodide ion or molecular iodine by H_2O_2 in solutions of SCN have been reported to produce a complex with the formula $I(SCN)_2^-$.[28] In another experiment, pulse radiolysis of aqueous solutions of SCN^- and I^- under oxidizing conditions led to the formation of $ISCN^-$.[29] When the product formed by LPO/H_2O_2/I^-/SCN^- system were investigated, Schlorke[27] *et. al*. identified ICN using ^{13}C NMR and GC-MS spectrometry as the main oxidation product. Whilst Bafort *et. al*. identified I_2SCN^- using ^{13}C NMR and ESI-Mass spectrometry as the main product.[29]

1.2.1 Peroxidase-generated antimicrobials in exocrine fluids

Exocrine glands such as salivary glands, mammary glands, and mucous glands secrete substances or fluids through a duct opening in a gland to an epithelial surface.[30, 31] Examples of exocrine fluids are saliva, milk, tears, mucous, and other digestive juices. These fluids play a crucial role in host defence mechanisms against microbial infection.[32] Human defensive peroxidases which use hydrogen peroxide as an oxidant of halides and pseudohalides to produce more effective antimicrobials are common to all exocrine fluids.[21]

The two main peroxidase systems operating in the oral cavity are salivary peroxidase (SPO) and myeloperoxidase (MPO). SPO differs from MPO because it originates from salivary glands while MPO is derived from neutrophils entering the oral cavity during inflammatory response.[21, 33] Estimates indicate that approximately 75% of the peroxidase activity in mixed saliva is due to MPO, with the remaining 25% activity attributed to SPO.[20] SPO activity is mainly associated with the soluble portion of the saliva, whereas most of the MPO activity is related to the non-soluble part.[20] SPO and MPO concentrations are said to typically range from 2 to 13 ug ml^{-1} in the saliva of healthy adult individuals.[21, 33]

The amount of available hydrogen peroxide (H_2O_2) determines the oxidation products in the oral cavity. Oral streptococci constitute a significant source of H_2O_2 during the fermentation of lactic acid in the oral cavity.[34, 35] Endogenous sources of H_2O_2 in the saliva include the dual-oxidase system from the salivary glands[36-38] and activated neutrophils during oxidative respiratory burst.[39] SPO can facilitate the oxidation of halides Br^-, I^- and SCN^- to hypo-pseudo-halides; OBr^-, OI^- and $OSCN^-$ respectively, while MPO can oxidise Br^-, I^-, SCN^- and Cl^-. MPO catalyses the oxidation of SCN^- approximately 1000 times faster than Cl^-.[40] It is expected, therefore, that comparable amounts of $OSCN^-$ and OCl^- are produced by the MPO system in saliva where SCN^- and Cl^- are present at concentrations of 0.1 – 3 mM and 20 mM respectively.[34, 35] Ashby *et al.* estimated the half-life of OCl^- in saliva-like conditions at less than 15 micro seconds due to its fast non-enzyme reaction with SCN^- to produce $OSCN^-$.[41, 42] It is thus deduced that $OSCN^-$ is the only dominant hypohalite of the SPO and MPO system in saliva.

1.2.2 The mucosa in the defence mechanism

The mammalian body is susceptible to infection by many pathogens, which must first contact the host and then establish a focus of infection to cause disease. These pathogens differ significantly in their behaviour, the structures of their surfaces, and means of pathogenesis, which therefore requires an equally diverse set of defensive responses from the host immune system.[43] Our bodies are continually exposed to microorganisms found in the environment, including pathogenic organisms excreted by contagious diseases. Contact with these microorganisms can occur through external or internal epithelial surfaces such as the

respiratory tract mucosa, gastrointestinal mucosa, insect bites, wounds, and direct contact between individuals.[43]

The mucosal surface in humans and animals is lined with epithelial cells and mucus-secreting cells, forming tight barriers separating the exterior surroundings from the interior compartments. It serves as a vital link between the host cells and their surroundings. Mucosal epithelial cells are vulnerable to microbial attacks as they are constantly exposed to external environments. They play an essential role in regulating mucosal immune responses by locally adapting microbial recognition, maintaining immune homeostasis, modulating antigen-presenting cells, and adaptive immune responses during the interaction of host and external pathogens.[44] Mucosal epithelial cells play a vital role in defending the host from pathogen invasion by secreting various antimicrobial compounds into the mucosal fluid, including mucins, defensins, lysozyme, nitric oxide, and others such as the generation of sIgA.[45, 46] These defence compounds generate a physical barrier on the mucosal surfaces and have direct antibacterial activity.

1.3 Antimicrobials from oxidation of SCN^-

Human defensive peroxidases such as; MPO, EPO, SPO and LPO are all capable of oxidizing SCN^- into various antimicrobial species.[47] While $OSCN^-$ is generally assumed an exclusive product of SCN^- oxidation with antimicrobial properties, many other potentially active chemical species are possible. The LPO–H_2O_2 system oxidizes SCN^- to $OSCN^-$ with a high rate of 2×10^8 M^{-1} s^{-1} at pH 7.0 (equation 8) relative to halogens, namely, bromide and iodide which react with second-order rate constants of $(4.1 \pm 0.1) \times 10^4$ M^{-1} s^{-1} and $(1.2 \pm 0.04) \times 10^8$ M^{-1} s^{-1} respectively.[48] The proposed pathways for the LPO-catalysed oxidation of SCN^- is indicated by the stepwise formation of the product in equations five and six. The deprotonated species ($OSCN^-$) is in equilibrium with hypothiocyanous acid (HOSCN) with a p*K*a value of 5.3 (equation 7).[49]

$$H_2O_2 + 2SCN^- + 2H^+ \xrightarrow{\text{peroxidase}} (SCN)_2 + 2H_2O \quad (\mathbf{5})$$

$$(SCN)_2 + H_2O \rightarrow HOSCN + H^+ + SCN^- \quad (\mathbf{6})$$

$$HOSCN \rightleftharpoons H^+ + OSCN^- \quad (\mathbf{7})$$

OR

$$H_2O_2 + SCN^- \xrightarrow{\text{peroxidase}} OSCN^- + H_2O \quad (\mathbf{8})$$

Whether the reaction proceeds *via* the direct pathway (equation 8) or *via* the formation of thiocyanogen $(SCN)_2$ (equation 5), and its rapid hydrolysis (equation 6), $OSCN^-$ remains the dominant product at physiological pH.[49] When the concentration of H_2O_2 is high, other highly reactive, short-lived antimicrobial products have been postulated at neutral pH as reported by Pruitt and co-workers.[50] These higher oxyacids include cyanosulfite (O_2SCN^-) (equation 9) and cyanosulfate (O_3SCN^-) (equations 10), which potentially are more effective microbial inhibitors than $OSCN^-$.

$$OSCN^- + H_2O_2 \longrightarrow O_2SCN^- + H_2O \quad (\mathbf{9})$$

$$O_2SCN^- + H_2O_2 \longrightarrow O_3SCN^- + H_2O \quad (\mathbf{10})$$

1.4 Antimicrobials from oxidation of I^-

Depending on the pH and the halide concentration, the LPO – H_2O_2 system oxidizes I^- in to a mixture of iodine species such as $I_2/I_3^-/I_2OH/HOI$.[51] HOI is the most effective electrophilic iodine (I^+) antimicrobial under physiological conditions.[52, 53] Hypoiodous acid (HOI, pKa = 10.6) formation occurs rapidly with a second-order rate constant of $1.2 \times 10^8\ M^{-1}\ s^{-1}$ at pH 7.0. Due to the complexity of iodine chemistry, there is no specific agent that was fully reported for killing bacteria in the peroxidase/I^- system but rather a mixture of iodine species.[25]

Oxidation of iodide can take place in the presence or absence of a peroxidase. In the absence of a peroxidase, the reaction of iodide with hydrogen peroxide produces short-lived intermediates such as HOI/OI^- and some observable intermediates such as I_2 and I_3^-.[54] This reaction involves two reactions. The first reaction produces HOI and the second fast reaction produces free iodine (equation 11 and 12). Triiodide is also present in solution where it is in equilibrium with I_2 and I^- (equation 13).[55]

$$H_2O_2 + I^- + H^+ \longrightarrow HOI + H_2O \quad (\mathbf{11})$$

$$HOI + I^- + H^+ \rightleftharpoons I_2 + H_2O \quad (\mathbf{12})$$

$$I_2 + I^- \rightleftharpoons I_3^- \quad (\mathbf{13})$$

For the inorganic iodine-water based system, a number of iodine species are produced in which the equilibria are controlled by the H^+ and I^- concentrations. This implies that the pH

and increased iodide have an impact on the concentrations and the species that are present (equation 14 to 22).[47,48]

$I_2 + H_2O \rightleftharpoons HOI + I^- + H^+$ (**14**)

$HOI \rightleftharpoons OI^- + H^+$ (**15**)

$I_2 + I^- \rightleftharpoons I_3^-$ (**16**)

$HOI + H^+ \rightleftharpoons H_2OI^+$ (**17**)

$I_3^- + I_2 \rightleftharpoons I_5^-$ (**18**)

$2I_3^- \rightleftharpoons I_6^{2-}$ (**19**)

$OI^- + I^- + H_2O \rightleftharpoons HI_2O^- + OH^-$ (**20**)

$HI_2O^- \rightleftharpoons I_2O^- + H^+$ (**21**)

$3HOI \rightleftharpoons IO_3^- + 2I^- + 3H^+$ (**22**)

At a pH of six or less and with excess iodide, only I_2 and I_3^- have the maximum oxidation power in the most usual instance. At pH 8 to 9, with lower iodide concentration, HOI accounts for most of the oxidation capacity, and with excess iodide, I_6^{2-} and I_5^- are major products. At a pH that is greater than 10, H_2OI^+, OI^- and HI_2O^- are important. At pH < 4, IO_3^- can act as an oxidant in a form of HIO_3, but has no oxidative activity in neutral and basic pH conditions.[56, 57]

In the presence of a peroxidase, the antimicrobial activity of the peroxidase/H_2O_2/I^- system is possible due to the oxidation of I^- to I_2 which is followed by rapid iodination of cell components (equation 23).[58]

$H_2O_2 + 2I^- + 2H^+ \xrightarrow{\text{peroxidase}} I_2 + 2H_2O$ (**23**)

1.5 Antimicrobials from combination of SCN^- and I^-

In a system containing a mixture of SCN^- and I^- as substrates, a competition for oxidation *via* non-enzymic and peroxidase-catalysed reactions occurs. This system is reported in chemical literature as behaving like the well-documented reaction between I_2 and SCN^- in aqueous solutions which produces the inter-pseudo-halogen; cyanogen iodide (ICN). In neutral and slightly basic solutions, ICN is produced according to equation 24.[26, 59]

$$4I_2 + SCN^- + 2H_2O \longrightarrow SO_4^{2-} + 7I^- + 8H^+ + ICN \quad (\mathbf{24})$$

Griffith and Mckeown reported that dilute solutions of strong acids containing I^-/I_2 and SCN^- resulted in the formation of a yellow unstable complex, diiodothiocyanate (I_2SCN^-) using different buffers.[60, 61] Schoneshbfer and Henglein studied intermediate species that formed during pulse radiolysis of aqueous solutions containing a mixture of I^- and SCN^-. The reaction produced a mixture of complexes presumably I_2SCN^- and $I(SCN)_2^-$.[62] In aqueous solutions, both species are unstable and quickly decomposes to ISCN (equations 25 and 26).

$$SCN^- + I_2 \rightleftharpoons I_2SCN^- \rightleftharpoons ISCN + I^- \quad (\mathbf{25})$$

$$I_2 + 4SCN^- \rightleftarrows 2I(SCN)_2^- \quad (\mathbf{26})$$

It has also been demonstrated that a mixture of I^- and SCN^- is oxidized by H_2O_2 to an unstable complex with a probable formula; dithiocyanate iodide [$(I(SCN)_2^-)$] in aqueous media (equations 27 and 28).

$$H_2O_2 + I^- + SCN^- + 2\,H^+ \longrightarrow I(SCN)_2^- + 2H_2O \quad (\mathbf{27})$$

$$H_2O_2 + I_2 + 4SCN^- + 2H^+ \longrightarrow 2I(SCN)_2^- + 2H_2O \quad (\mathbf{28})$$

Schlorke and co-workers studied the oxidation products of the LPO/H_2O_2/I^-/SCN^- system.[27] They used ^{13}C NMR and gas chromatography-mass spectrometer to show that when I^- and SCN^- are in excess, ICN was generated.[27] This product was detected in systems where LPO was replaced with MPO and in enzyme-free systems. Furthermore, Bafort and co-workers have reported the presence of I_2SCN^- in the LPO/H_2O_2/I^-/SCN^- system by ^{13}C NMR and ESI-mass spectrometry.[29] They indicated that the variability of the oxidation products depends upon the ionic strength and the pH of the solution.

1.6 Problem statement

The dawning of the antibiotic era in the middle of the 20th century gave hope that the spread of infectious diseases was finally ending. During this time, antibiotics were considered the 'wonder drug' to fight against disease-causing microbes. The genius of antibiotics working is their ability to selectively target microbes while not harming the host. Antibiotics have since their inception transformed medicine and saved millions of lives.[29, 63-66] The rapid worldwide

emergence of resistant bacteria is undermining the efficacy of antibiotics and simultaneously threatening lives. The antimicrobial resistance (AMR) crisis is mainly attributed to the overuse and misuse of antibiotics.[64, 65]

The global community recognises antimicrobial resistance as a growing and serious threat to human health. The cost to human life associated with AMR is currently estimated at more than 700 000 deaths per year worldwide.[67] It is further projected AMR will cost approximately 10 million lives and about 100 trillion US dollars per year by 2050.[67]

Important organizations like World Health Organization (WHO) and the Centre for Disease Control and Prevention (CDC) have proposed a global plan of action to combat antimicrobial resistance. The strategy includes renewed effort on research aimed at finding new effective antimicrobial agents.

The role of oxidants such as HOSCN, HOCl and HOBr in human inflammatory diseases is widely reported, while the possible antimicrobial action of iodine-based compounds is generally ignored. This is because iodine concentration in physiological fluids is often assumed to be low. However, iodine concentration in saliva and other mucosal fluids is relatively higher compared to other non-thyroidal physiologic fluids. Thermodynamics dictates that at physiological conditions HOSCN oxidizes I^- to produce I_2 which further reacts with SCN^- to give ICN. ICN is a more potent antimicrobial agent that is active against bacteria, fungi and viruses than HOSCN. Studies have shown that iodine (I^- and I_2) can react with thiocyanate *via* uncatalyzed or peroxidase-catalysed reactions to form inter-pseudo-halogens that are more cytotoxic.[27, 29] In this study, products of the reaction between iodine and thiocyanate are identified and their activity tested against two bacteria (*E. Coli* and *E. faecalis)*.

1.7 Aims and Objectives

1.7.1 Aims

- To identify and quantify the iodine-based antimicrobials produced through a non-enzymic pathways in the presence of SCN^- and I_2.
- To test the efficacy of the antimicrobial products on two bacteria (*Escherichia coli* and *Enterococcus faecalis*) on normal strains (ATCC) and MRD strains (GCE).

1.7.2 Objectives

- To follow the reaction between I_2 with SCN^- and identify the products using stopped flow and UV/Visible spectrophotometer.
- To synthesise and characterise the main product that forms when SCN^- reacts with I_2, using LC-MS spectrometry, ^{13}C NMR spectroscopy, and UV-Visible spectroscopy.
- To evaluate the antimicrobial properties of the products on two different bacteria (*E. Coli* and *E. faecalis*) on normal strains (ATCC) and MRD strains (GCE).

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Chapter 2: Experimental Methods

2.1 General Information

All chemicals utilised, unless otherwise stated, were purchased from Sigma-Aldrich and they were used as received without further purification. The solutions were prepared using acetonitrile, phosphate buffer and sodium acetate buffer unless stated. The chemicals used in this study; iodine (I_2, 99.8%), sodium thiocyanate, (NaSCN, 99%), acetonitrile (CH_3CN 99%), sodium phosphate monobasic dihydrate ($NaH_2PO_4.2H_2O$, 99%), and sodium phosphate dibasic (Na_2HPO_4, 99%), sodium hydroxide pallets (NaOH, 98%), hydrochloric acid (HCl, 32%), cyanogen iodide (ICN, 99%), were all used as received from Sigma Aldrich.

2.2 Sample preparation

2.2.1 Preparation of iodine (I_2) solution

Two different methods were used to prepare iodine solutions.

In the first method iodine granules were dissolved in a 100 mL Erlenmeyer flask with a portion of distilled water. The solution was stirred for 24 hours at room temperature in the dark.[59] The dissolved iodine was filtered and the concentration was determined spectrophotometrically by measuring the absorbance at 460 nm.

In the second method small amounts of iodine granules were dissolved in acetonitrile. The dissolved iodine was then filtered and the concentration of the iodine solution was determined spectrophotometrically by measuring the absorbance at 460 nm, I_2 (λ_{max} = 458/460 nm, $\varepsilon = 703 \times 10^4\ M^{-1}\ cm^{-1}$).[60]

2.2.2 Preparation of SCN^- stock solution

Sodium thiocyanate stock solution (30 mM) was prepared in two different solvents, distilled water and acetonitrile. 24.32 mg salt of NaSCN was dissolved in 10 mL distilled water (pH 3,6 or 7,4) or 10 mL acetonitrile. The stock solution was used to prepare desired concentrations by diluting with distilled water.

2.2.3 *In situ* generation of Diiodothiocyanate (I_2SCN^-) from the reaction of I_2 and SCN^-

I_2SCN^- was formed by mixing 0.1 mM of I_2 with 0.1 mM of SCN^- in a 1:1 ratio, prepared in distilled water at a pH of 3.6. The rate of formation of I_2SCN^- was followed by the stopped-flow spectrophotometer at its maximum absorbance wavelength of 300-309 nm, I_2SCN^- (ε_o = 4.75 x 10^4 M^{-1} cm^{-1}).[61, 62]

I_2SCN^- was formed by mixing 0.1 mM of I_2 with 0.1 mM of SCN^- in a 1:1 ratio, prepared in CH_3CN. The formation was followed using UV-Visible spectrophotometer at its maximum absorbance wavelength of 305 nm.[62] The solution prepared in CH_3CN was kept in the fridge at a temperature of ±4 °C to maintain stability.

2.2.4 *In situ* generation of I_2SCN^- from the reaction of ICN and SCN^-

Cyanogen Iodide (ICN) solution (9.0 mM) was prepared by dissolving 6.98 mg in 5 ml distilled water (pH 7,4). I_2SCN^- was formed by mixing ICN with SCN^- in a 1:1 ratio.[20] The rate of formation for the reaction was followed by UV-Vis spectrophotometer at 305 nm. LC-MS and ^{13}C NMR spectrometers were used to characterise the presence of I_2SCN^-.[20]

2.3 Strain details and handling of cultures of *Escherichia coli* (*E. coli*) and *Enterococcus faecalis* (*E. faecalis*) strains.

3.2.1 *Escherichia coli* strains

Three different *Escherichia coli* strains were used. The first strain was obtained from the American Type Culture Collection, *Escherichia Coli* (ATCC 25922). The other two *E. coli* bacterial strains (FP5 and FP29) were environmental isolates multidrug resistant (MDR) type. They were isolated from farm pigs and were kindly provided by Tracy Leigh Beetar-King (Microbiology, School of Life Sciences, University of KwaZulu-Natal). Bacterial strains were cultured on Nutrient agar (NA) plates and incubated at 37 °C for 24 hours.

2.3.2 *Enterococcus faecalis* strains

Three different *Enterococcus faecalis* strains were used. The first strain used was obtained from the American Type Culture Collection, *Enterococcus faecalis* (ATCC 29212). The other two *Enterococcus faecalis* bacterial strains (GCE8 and GCE22) were environmental isolates

kindly provided by Tracy Leigh Beetar-King (Microbiology, School of Life Sciences, University of KwaZulu-Natal). These strains represented MDR isolates and were isolated from clinical effluent Bacterial strains, they were cultured on Brain Heart Infusion (BHI) agar plates and incubated at 37 °C for 24 hours.

2.3.3 Handling of cultures

A single colony from Nutrient agar (NA) plates and Brain Heart Infusion (BHI) agar plates, were aseptically transferred to 10 mL of Nutrient broth (NB) and Brain Heart Infusion (BHI) broth. Shaker incubation was used to mix at 100 rpm for 24 hours at a temperature of 37 °C, until the cultures reached the stationary phase. Phase contrast and bright field microscopy of NA and BHI agar were utilized to confirm purity. 1 mL of cell suspension culture with an OD of about 0.600 at 600 nm was kept at −80 °C in BHI in the presence of 20% glycerol for storage.

2.3.4 Antimicrobial susceptibility testing by the Disk diffusion method

Evaluations of the antimicrobial activity of I_2SCN^- were performed using the disk diffusion method.[63, 64] BHI agar plate surfaces were inoculated by spreading a volume of 100 µl of all *E. faecalis* strains at 0.5 McFarland ($1 – 2 \times 10^8$ CFU/mL) evenly over the entire agar surface. Sterile filter disks were aseptically transferred to the plate with the use of a sterile tweezer, then 20 µl of I_2SCN^- extract was pipetted onto the disk. The plates were incubated at 37 °C for 18 to 24 hours before recording diameter of the diffusion disk (zones of inhibition) (Figure 2.1). Acetonitrile was used in the extraction process and thus was used as the blank. The measurement of the diameter of the zone of inhibition *in-situ* produced by I_2SCN^- was done in triplicates.

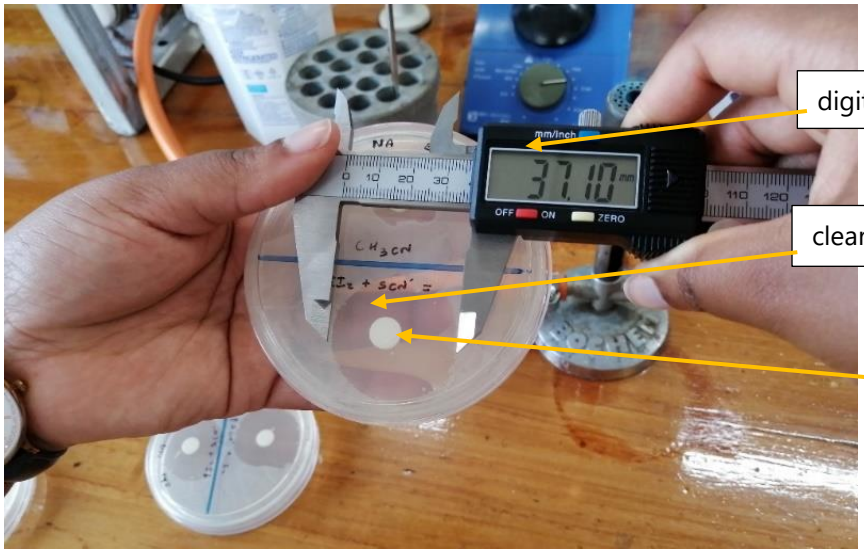

Figure 2. 1: Measurements for Disk diffusion method of the zone of inhibition.

2.3.5 Growth kinetics of *E. coli* and *E. faecalis* isolates.

The cultured bacterial stocks (in glycerol) of *E. coli* strains (ATCC 29522, FP 5 and FP 29) and *E. faecalis* (ATCC 29212, GCE 8 and GCE 22*)* were used by streaking them out on NA plates and BHI agar plates, respectively. The plates were incubated upside down at 37 °C for 24 hours. 25 ml of sterile NB and BHI broth were aseptically inoculated using a single bacterial colony from each strain in a 100 mL Erlenmeyer flask and incubated on a shaker for 24 hours at 37 °C and 100 rpm. Cultures were inoculated in 100 mL of sterile NB and BHI broth which was pre-heated to 37 °C in a 500 mL Erlenmeyer flask, to an OD 600 baseline of 0.1 – 0.2. The OD 600 values were then recorded every 30 minutes for 6 to 7 hours or until the growth reached the stationary phase. The last reading was taken 24 hours after the stationary phase.

The bacterial growth kinetics were analysed at a final volume of 35 mL of liquid culture, with three different concentrations of 0.10, 0.25, and 0.50 mM of I_2SCN^- for all the strains. The bacterial growth was measured spectrophotometrically by following the absorbance. The optical density (OD) was calculated at 600 nm.

2.3.6 Data and statistical analysis of *E. coli* and *E. faecalis* isolates

The data collected from UV-Visible Mini 1240, was analysed using Excel statistical software. The optical density measurements were computed using data collected in triplicates. The

mean and standard deviation were also calculated from the studies of the antimicrobial activities.

2.4 Instrumentation

2.4.1 UV-Vis Spectrophotometer

A Varian Cary 100 Bio UV-Visible spectrophotometer and Cary 3500 UV-Vis Spectrophotometer with 1 cm path length quarts' cell was used to identify and quantify I_2SCN^- (λ_{max} = 300 nm, ε = 4.75 x 10^4 M^{-1} cm^{-1}) and Iodine (λ_{max} = 458/460 nm, ε = 703 M^{-1} cm^{-1}).

For kinetic growth of *E. coli* and *E. faecalis* isolates, Optical density (OD600) of each strain was measured spectrophotometrically using a UV-Visible spectrophotometer (UV Mini 1240, Shimadzu, Kyoto, Japan) and a 1 cm path length quartz cell.

2.4.2 pH Measurements

pH measurements were performed with a Metrohm 827 pH meter. The pH-meter was calibrated with standard pH reagents at pH 4.0; 7.0 and 10.0.

2.4.3 Stopped-flow Spectrophotometer

An applied Photophysics SX-20 MV stopped-flow spectrophotometer equipped with a xenon arc lamp and a photodiode detector covering the spectral range of 190 – 730 nm was used for the kinetic measurements of the reaction of I_2 with SCN^-. The temperature was controlled throughout all kinetic experiments to within ±0.1 °C using a coupled temperature control unit.

2.4.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

The products of the reaction between I_2 and SCN^- were characterised by ionization spectrometry using a Shimadzu LC-MS, ESI-quadrupole-2020 instrument in both positive and negative ionization modes.

2.4.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

A Bruker Avance III 400 or Bruker Avance III 500 spectrometer was used to record ^{13}C NMR spectra at frequencies of 100 MHz or 125 MHz using either a 5 mm BBOZ probe or a 5 mm

TBIZ probe. All data were recorded at 303 K. The data was analysed with Topspin, a Bruker software.

2.5 Software Data Analysis

The polychromatic data from the stopped-flow spectrophotometer were processed using the Pro-Data Viewer software supplied with the stopped-flow spectrophotometer and analysed using the program SPECFIT/32 version 2.10 for MS-DOS (Spectrum Software Associates, Chapel Hill, NC, USA), a multivariate data analysis program.

The polychromatic data from UV-Visible and monochromatic data from stopped-flow spectrophotometers were analysed using the Origin 9.1® graphical analysis software package (Northampton, Massachusetts, USA).

2.6 References

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Chapter 3: Results and Discussion

3.1 Synthesis and characterization of diiodothiocyanate (I_2SCN^-)

The formation of the inter-pseudo-halogen diiodothiocyanate (I_2SCN^-) in mixtures of I_2/I^- with SCN^- especially in the presence of oxidizing agents such as H_2O_2 or IO_3^- has been proposed.[19, 61, 65] In the present study, I_2SCN^- was generated by the reaction of I_2/I^- with SCN^- and also the reaction of ICN with SCN^- that was previously reported by Bafort and co-workers.[20]

3.1.1 Reaction of I_2/I^- with SCN^- in acidic distilled water

The reaction of I_2/I^- with SCN^- in distilled water at pH 3.6 was followed spectrophotometrically using the stopped flow instrument coupled with a photodiode-array detector in the range 200 to 600 nm. Given the complexity of iodine chemistry in aqueous solutions, the reaction can produce a mixture of iodide-based chemical species.[51, 53, 59, 66, 67] The reaction was found to be highly dependent on the pH and mole ratios of the two reactants. The mixing of I_2/I^- with SCN^- in distilled acidic water was accompanied by the formation of a yellow-coloured product. The spectral changes observed for the reaction is shown in Figure 3.1. The spectra exhibit absorption bands at 250, 276, 299, 356 and 460 nm, with an isosbestic point at 435 nm, which is similar to the spectrum of I_2SCN^- in MeCN that was previously reported.[62] Absorbance bands below 300 nm are not reliable for accurate data evaluation due to interferences from multiple species absorbing in this region, such as ICN, $OSCN^-$, I^-.[26, 68-70]

Under the experimental conditions studied here, the I_2SCN^- complex is likely to be formed simultaneously with triiodide (I_3^-). The coexistence of I_2SCN^- and I_3^- in solution presents challenges for characterization as both species absorb in the range 290 – 400 nm. The characteristic band of iodine (I_2) in aqueous solutions at 460 nm is observed showing a slight decrease in intensity with time. The presence of an isosbestic point at 435 nm provides strong evidence for the presence of only two absorbing iodine species, namely I_2 and I_2SCN^-.

The increase in absorbance at 299 nm and 356 nm represents the formation of I_2SCN^- and is favoured when $[I_2] > [SCN^-]$.

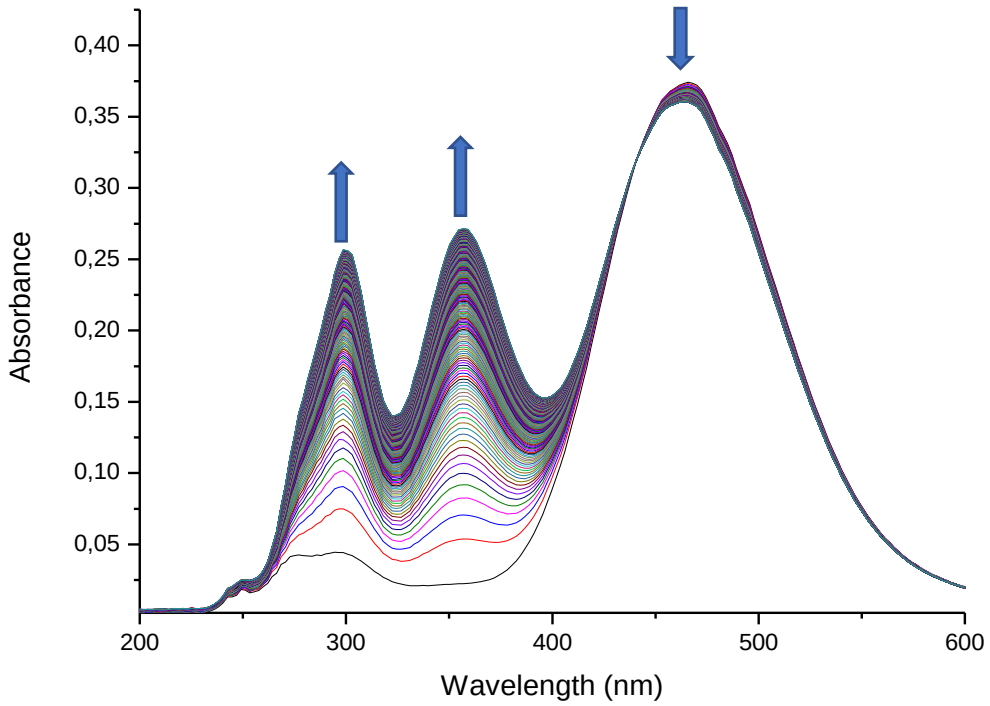

Figure 3.1: Spectral changes for the reaction of I_2 (0.1 mM) and SCN^- (0.01 mM) monitored for 60 seconds on the stopped flow spectrophotometer in acidic media (pH = 3.6).

Figure 3.2 illustrate changes in absorbance observed when $[SCN^-] > [I_2]$. Upon mixing 0.1 mM (I_2) with 1.0 mM (SCN^-) in acidified distilled water, a strong absorption band at 355 nm increasing with time is observed. This band represents the formation of I_3^-. The absorbance at 308 nm increases slightly with time. A clear isosbestic point at 435 nm confirms the presence of two absorbing iodine species, namely I_2 and I_3^-. The band at 460 nm decreases with time, characterising the depletion of I_2.

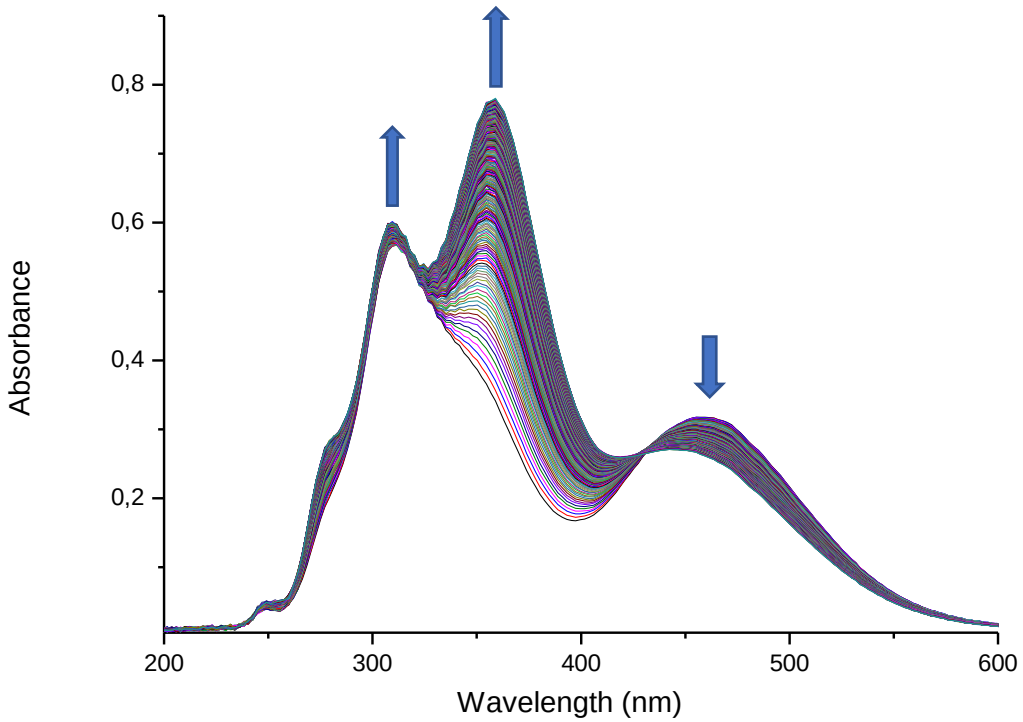

Figure 3.2: Spectral changes for the reaction of I_2 (0.1 mM) and SCN^- (1.0 mM) monitored for 60 seconds on the stopped flow spectrophotometer in acidic media (pH = 3.6).

The absorption spectra observed when equimolar solutions of I_2 (0.1 mM) and SCN^- (0.1 mM) were mixed are shown in Figure 3.3. Under these conditions, I_2SCN^- is expected to co-exist with I_3^- in solution. The spectra exhibit three strong absorptions bands at 305 nm, 356 nm, and 460 nm. The absorption band at 305 nm and 356 nm represents I_2SCN^- + I_3^- and it increases with time. Whilst the absorption band at 460 nm represents I_2 and it decreases with time.

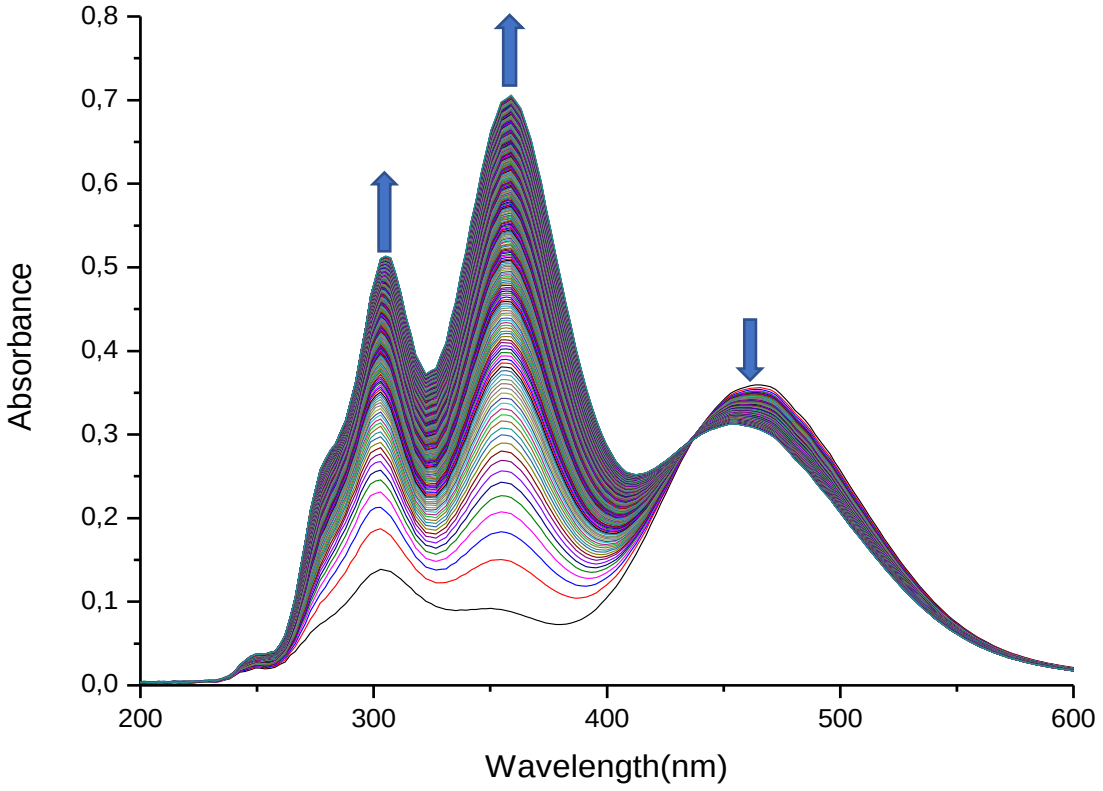

Figure 3.3: Spectral changes for the reaction of I_2 (0.1 mM) and SCN^- (0.1 mM) monitored for 60 seconds on the stopped flow spectrophotometer in acidic media (pH = 3.6).

The presence of I_2SCN^- and I_3^- as main products under equimolar conditions was confirmed by ESI-MS. A peak at 311 (m/z) confirms the presence of I_2SCN^- and a peak at 380 (m/z) confirms the presence of I_3^- (Figure 3.4). A peak for $I(SCN)_2^-$ was also detected at 242 (m/z). The presence of these three main species in aqueous solution is in agreement with literature data reported by Bafort.[20] Several other products such as ICN, ISCN and $(SCN)_2$ have been reported for the reaction of I_2 and SCN^-.[51, 67, 71] Some studies have reported the co-existence of I_2SCN^- and $I(SCN)_2^-$.[20, 62]

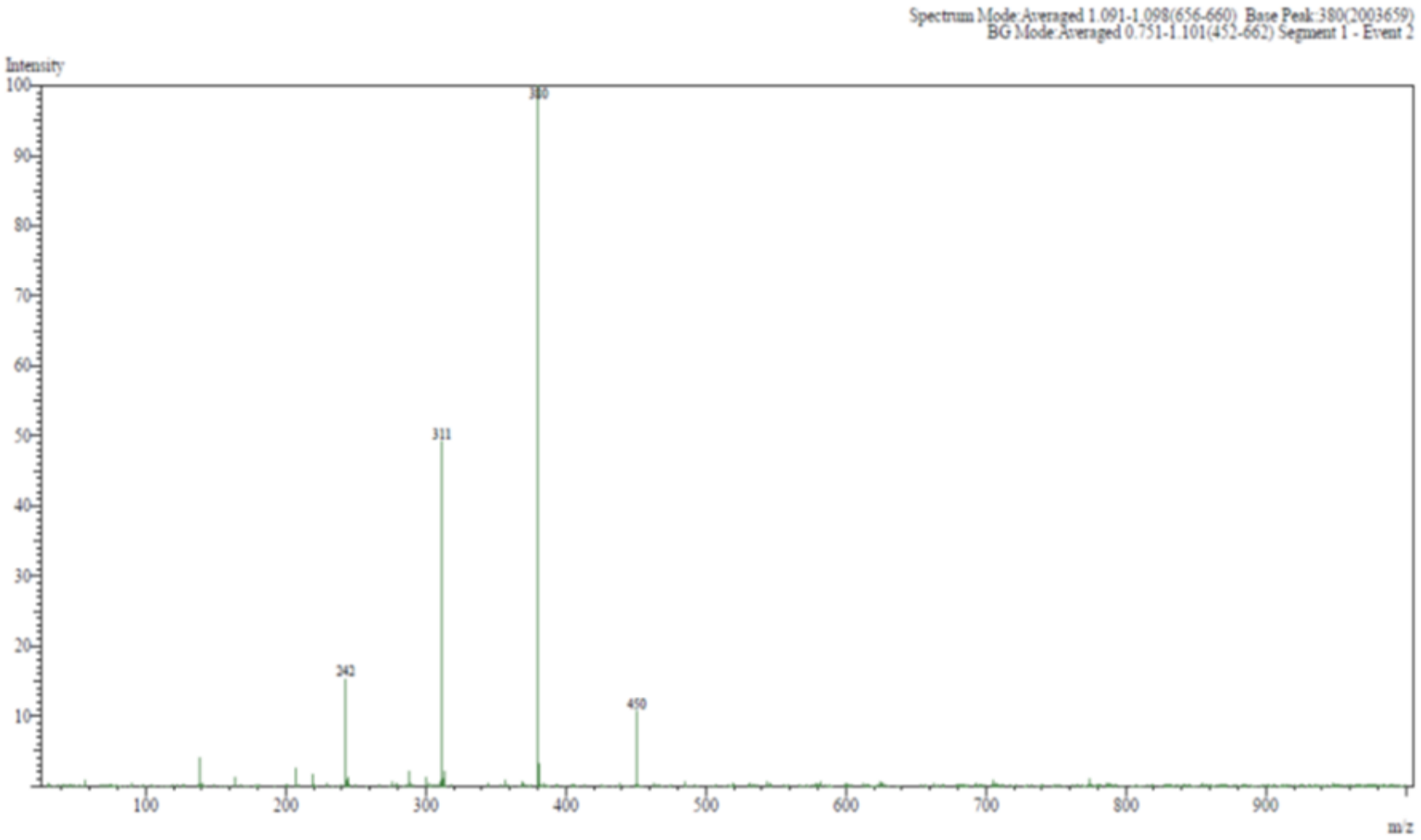

Figure 3.4: The mass spectrum of products formed by mixing 0.10 mM of I_2 and 0.10 mM of SCN^- at a pH of 3.6 in the negative ionization mode.

The reaction of I_2 with SCN^- was monitored using the diode-array on the stopped flow spectrophotometer in a single mixing mode. Figures 3.5 - 3.7 illustrates typical time-resolved kinetic traces for the reaction of I_2 (0.1 mM) and SCN^- (0.1 mM) at pH 3.6. The reactions were monitored at 460 nm (for I_2), 305 nm and 356 nm (for I_2SCN^-+ I_3^-).

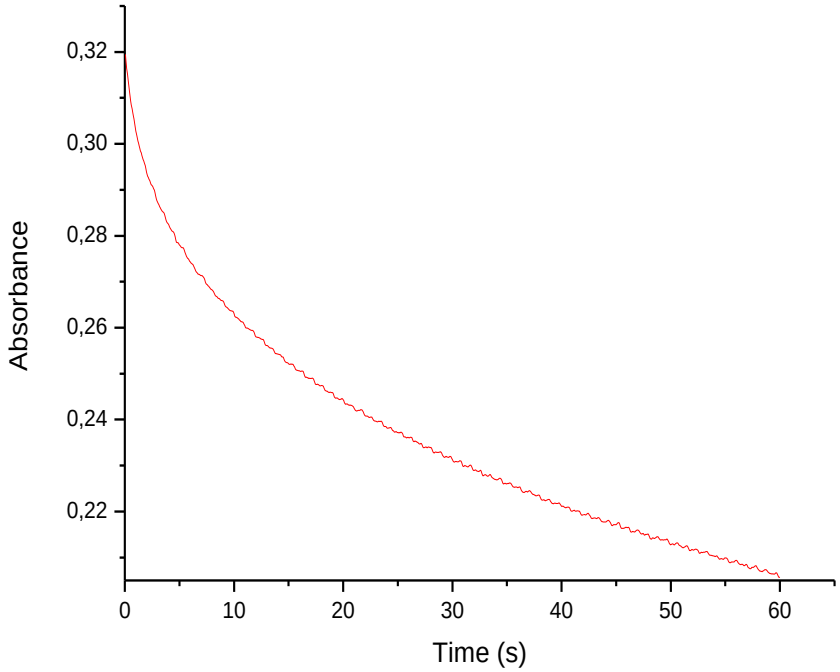

Figure 3.5: Stopped flow kinetic trace of I_2 (0.1 mM) and SCN^- (0.1 mM) for the disappearance of I_2 at 460 nm (pH of 3.6).

In order to monitor the reaction with the stopped flow technique, the reaction rate was minimized by employing equimolar reaction conditions. The kinetic trace at 460 nm (for the disappearance of I_2) shows the expected biphasic behaviour. The data shows that after 60 seconds, approximately 45 - 50% of I_2 has been depleted. The same type of plot was observed when the time was increased to 2000 s (see figure in the appendix).

The traces in figures 3.6 and 3.7 show the formation of I_2SCN^- + I_3^- at 305 and 356 nm respectively. The data exhibits first-order behaviour and the maximum formation of products achieved within 60 seconds.

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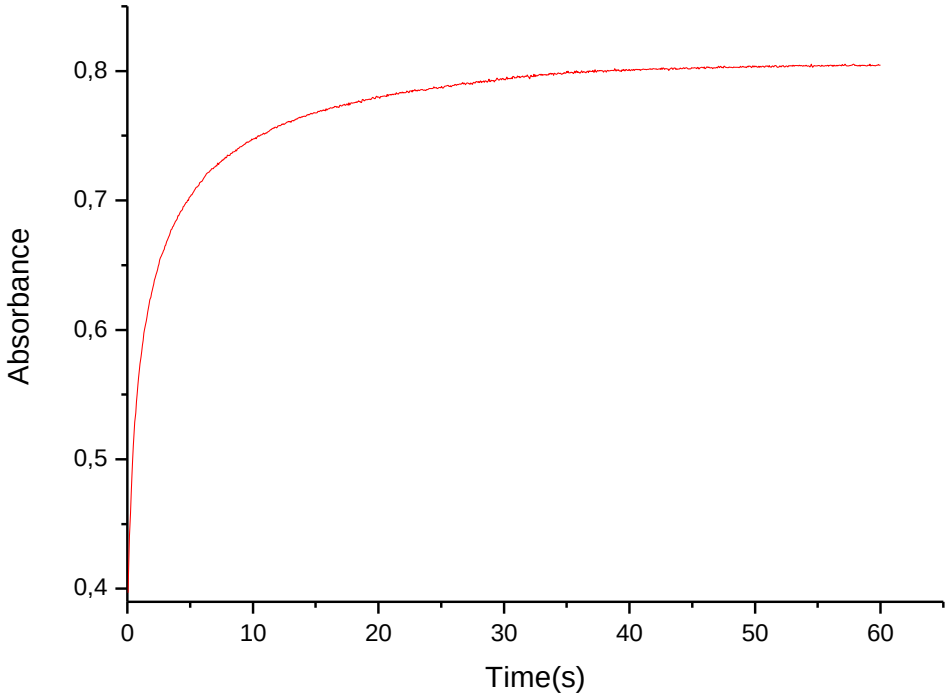

Figure 3.6: Stopped flow kinetic trace of I_2 (0.1 mM) and SCN^- (0.1 mM) at 356 nm for the appearance of I_3^- at a pH of 3.6.

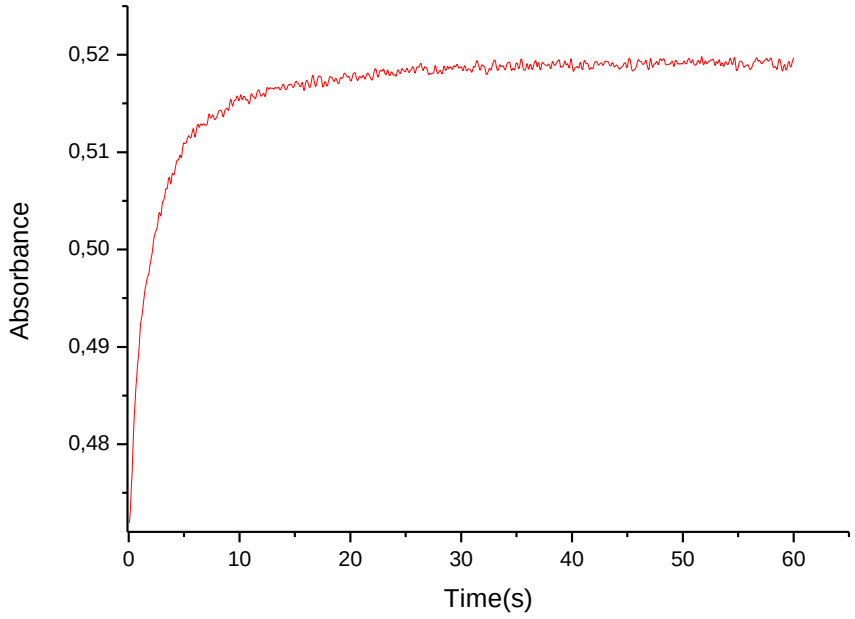

Figure 3.7: Stopped flow kinetic trace of I_2 (0.1 mM) and SCN^- (0.1 mM) at 305 nm for the appearance of I_2SCN^- at a pH of 3.6.

3.1.2 Reaction of ICN with SCN^- in distilled water

Bafort *et. al*. previously reported that ICN reacts with SCN^- to form I_2SCN^- using ^{13}C-NMR and mass spectrometry.[20] In the current study, the presence of I_2SCN^- in aqueous mixtures of ICN and SCN^- at pH 7.0 was confirmed with ^{13}C-NMR, mass spectrometry and spectrophotometrically using UV-Vis and stopped flow. The reaction produced a yellow product with a strong absorption band around 305 nm. The changes in the UV-Vis absorption spectra when ICN was reacted with SCN^- is shown in Figure 3.8. The spectra exhibit strong absorption bands at 225 and 305 nm, with an isosbestic point at 279 nm. The band at 225 nm, which indicates a decrease in absorbance represents ICN ($\varepsilon = 179\ M^{-1}cm^{-1}$) and I^- ($\varepsilon = 13400\ M^{-1}cm^{-1}$). The band at 305 nm, where the absorbance increases with time is attributed to I_2SCN^-.

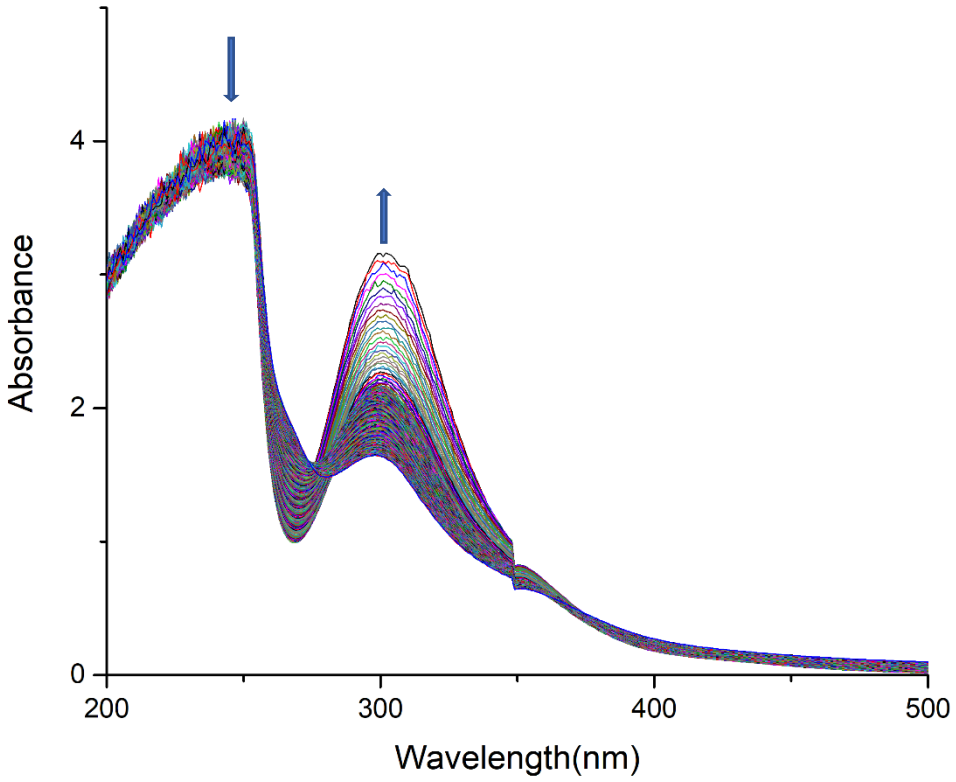

Figure 3.8: Spectral changes for the reaction of ICN (9.0 mM) and SCN^- (2.0 mM) monitored for 2100 minutes on the UV-Vis spectrophotometer in distilled water, pH 7.0.

The data for Figures 3.9 was collected at 305 nm and illustrates biphasic behaviour. The reaction is slow as indicated by the minute time scale. The time resolved trace at 305 nm shows an initial decrease in absorption, followed by an increase in absorption. This type of

kinetic behaviour is consistent with a stepwise reaction that forms an intermediate, followed by the stable final product (I_2SCN^-).

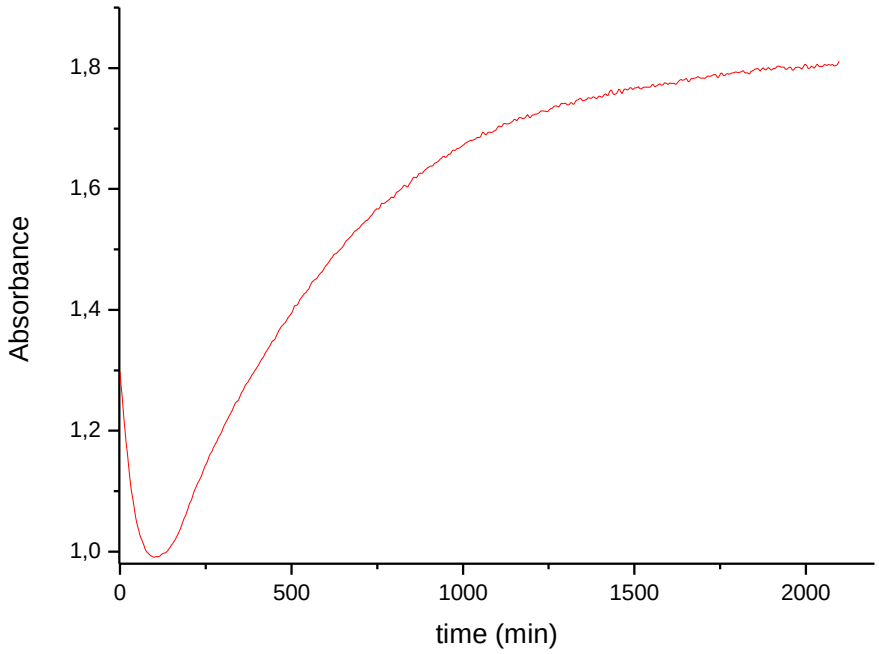

Figure 3.9: Kinetic trace at 305 nm for a reaction of ICN (9.0 mM) and SCN^- (2.0 mM) in distilled water, pH = 7.0.

Reaction mixtures of ICN with SCN^- at pH 7 were further studied by ^{13}C NMR spectrometry. In a previous study ^{13}C NMR spectroscopy was used to characterize products of the reactions of ICN with labelled $S^{13}CN^-$, I_2SCN^- was identified between 49.60 – 49.70 ppm and $I(SCN)_2^-$ at 103.64 ppm.[4] Figure 3.10, the ^{13}C NMR spectrum of ICN (355 mM) and SCN^- (79 mM) shows two signals at 49.40 ppm and 103.34 ppm. These signals which are shifted slightly upfield are attributed to I_2SCN^- (49.40 ppm) and $I(SCN)_2^-$ (103.34 ppm), respectively. It can be concluded from comparing the integral intensities of signals in ^{13}C NMR spectra that I_2SCN^- is a dominant species produced under the current experimental conditions.

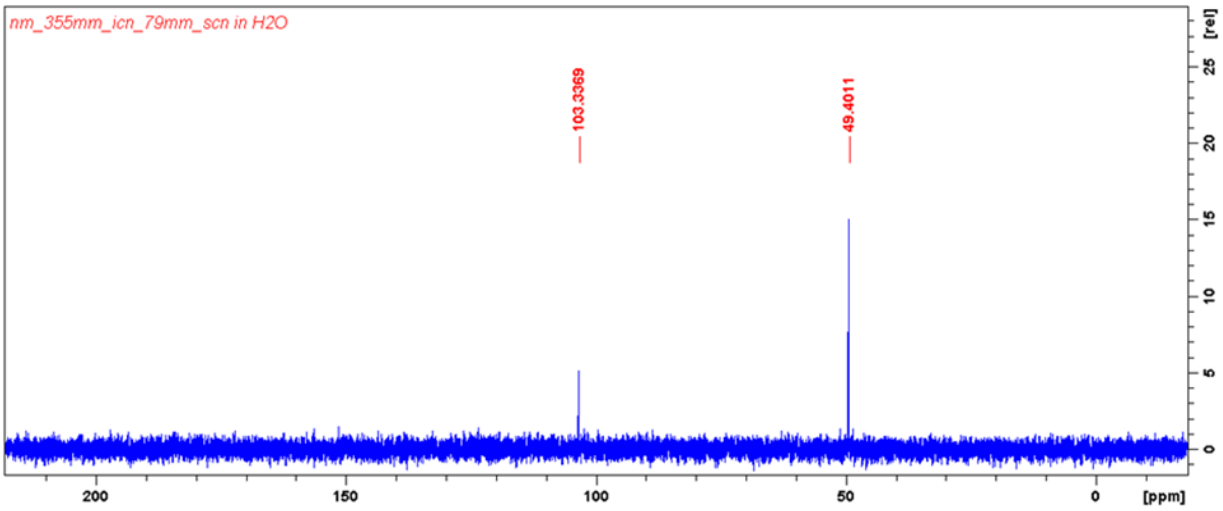

Figure 3.10: ^{13}C NMR spectrum of the reaction between ICN (355 mM) and SCN^- (79 mM) at a pH of 7.

Mass spectrometry was also used to confirm the presence of I_2SCN^- in aqueous mixtures of ICN with SCN^-. The ESI-MS spectrum (Figure 3.11) in the negative ionization mode shows a mass of 311 (m/z) attributed to I_2SCN^- as one of the dominant products. In contrast to the findings of Bafort *et. al.*, neither $I(SCN)_2^-$ at 242 (m/z) nor I_3^- at 380 (m/z) were observed as dominant products under the experimental conditions. Other undetermined fragments present in significant amounts were observed at 137 (m/z), 47 (m/z) and 27 (m/z).

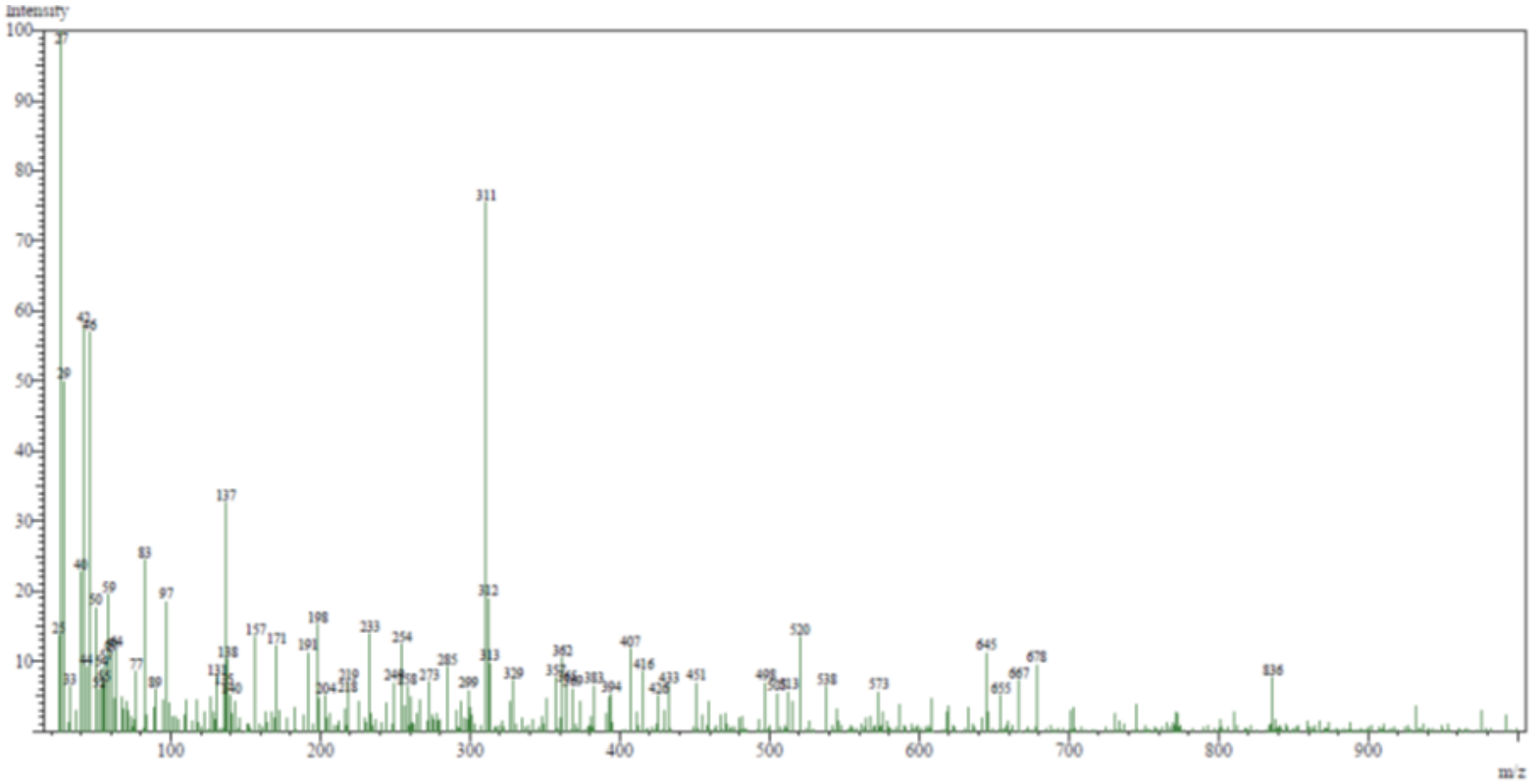

Figure 3.11: ESI-quadrupole mass spectrum of products formed by reacting 9.0 mM of ICN and 2.0 mM of SCN^- in distilled water at a pH of 7.0.

3.1.3 Reaction of I_2 with SCN^- in acetonitrile

Ashour *et. al*. reported that in acetonitrile (CH_3CN), I_2SCN^- forms rapidly and its intense yellow colour persists longer than in other solvents.[62] In aqueous solutions, the yellow colour disappears within minutes as I_2SCN^- hydrolyses to other stable products. The reaction of I_2 (0.1 mM) with SCN^- (0.1 mM) in acetonitrile produced an intensely yellow I_2SCN^- complex with an absorption maximum at 305 nm. In contrast to the spectra obtained in aqueous solution for I_2 and SCN^-, the UV-Vis absorption spectrum of I_2SCN^- in CH_3CN shows a small shoulder around 350 nm (Figure 3.12, blue). The shape of the absorption spectrum is consistent with the one reported in literature.[62]

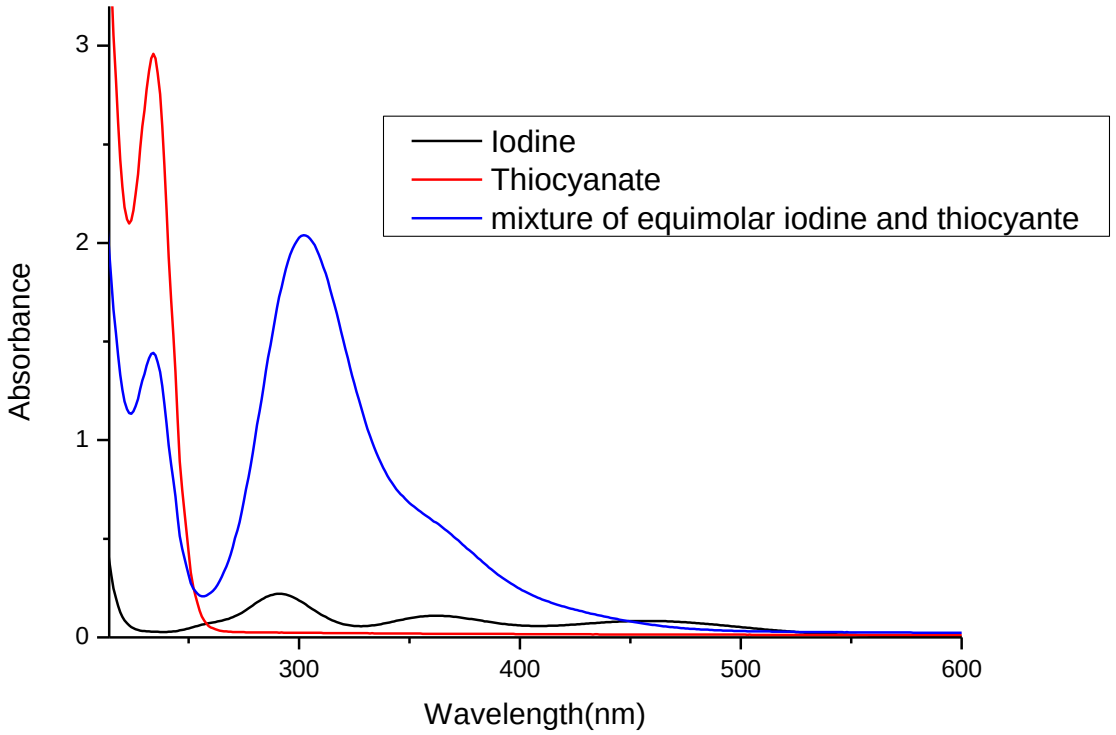

Figure 3.12: UV-Visible absorption bands of I_2 (black), SCN^- (red) and a reaction mixture of I_2 (0.1 mM) and SCN^- (0.1 mM) (blue) in acetonitrile.

The chemical stability of the I_2SCN^- complex in CH_3CN was determined by monitoring the loss of maximum absorption at 305 nm over time. Figure 3.13 shows a small decrease in absorbance at 305 nm over a period of 5 days at 15 °C (**A**) and 24 hours at 25 °C (**B**). From these results, we conclude that I_2SCN^- shows high stability in CH_3CN than in aqueous solutions.

The high stability of I_2SCN^- in CH_3CN allows for its use in other experiments such as the evaluation of its antimicrobial properties.

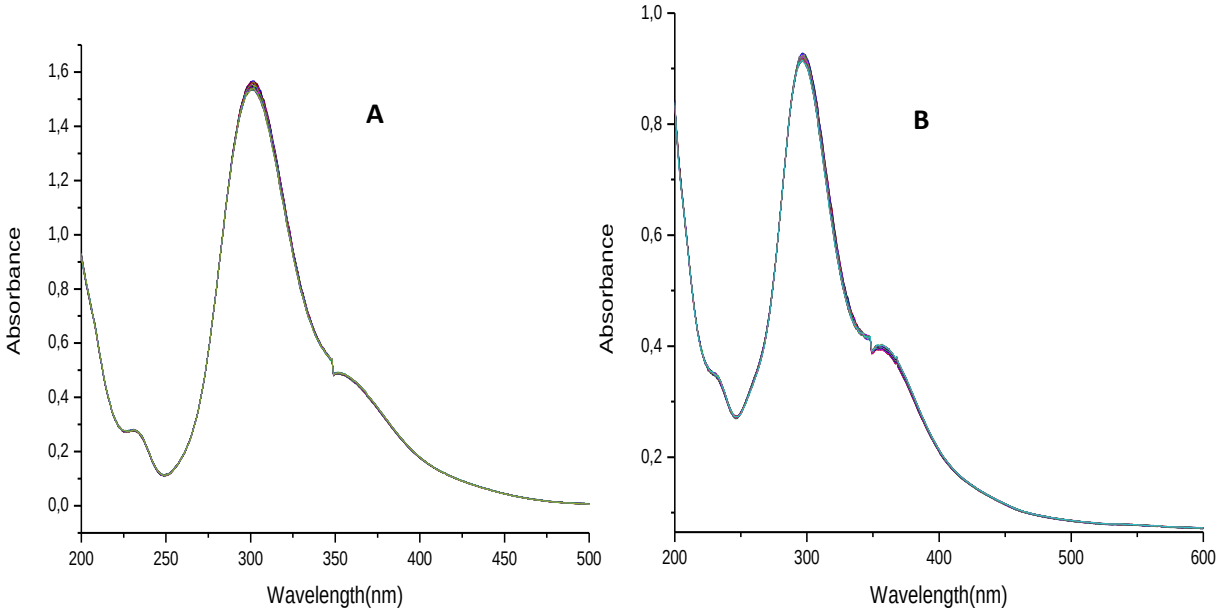

Figure 3.13: Changes in I_2SCN^- absorbance at 300 nm for the reaction of 0.1 mM I_2 and 0.1 mM SCN^- in CH_3CN collected over (**A**) 5 days at 15 °C and (**B**) 24 hours at 25 °C.

To further confirm the presence of Mixtures of I_2SCN^-, 0.1 mM I_2 and 0.1 mM SCN^- in CH_3CN was analysed by mass spectrometry. Figure 3.14 shows the spectrum obtained after mixing equimolar concentrations of I_2 and SCN^-. Two major products with signals at 311 (m/z) and 380 (m/z) corresponding to $I_2SCN^-$and I_3^- respectively were observed. The I_3^- signal is approximately ten times more intense compared to the I_2SCN^- signal after 5 days of reaction.

Previous studies have shown that the production of I_3^- in aqueous solutions of I_2/I^- and in I^-/H_2O_2/peroxidase systems have been tested for its antimicrobial activity.[72-75] The tests demonstrated that I_3^- has negligible antimicrobial properties. We thus do not expect the presence of I_3^- along with I_2SCN^- in acetonitrile solutions to interfere with the biological studies of I_2SCN^- against *E Faecalis* and *E coli*.

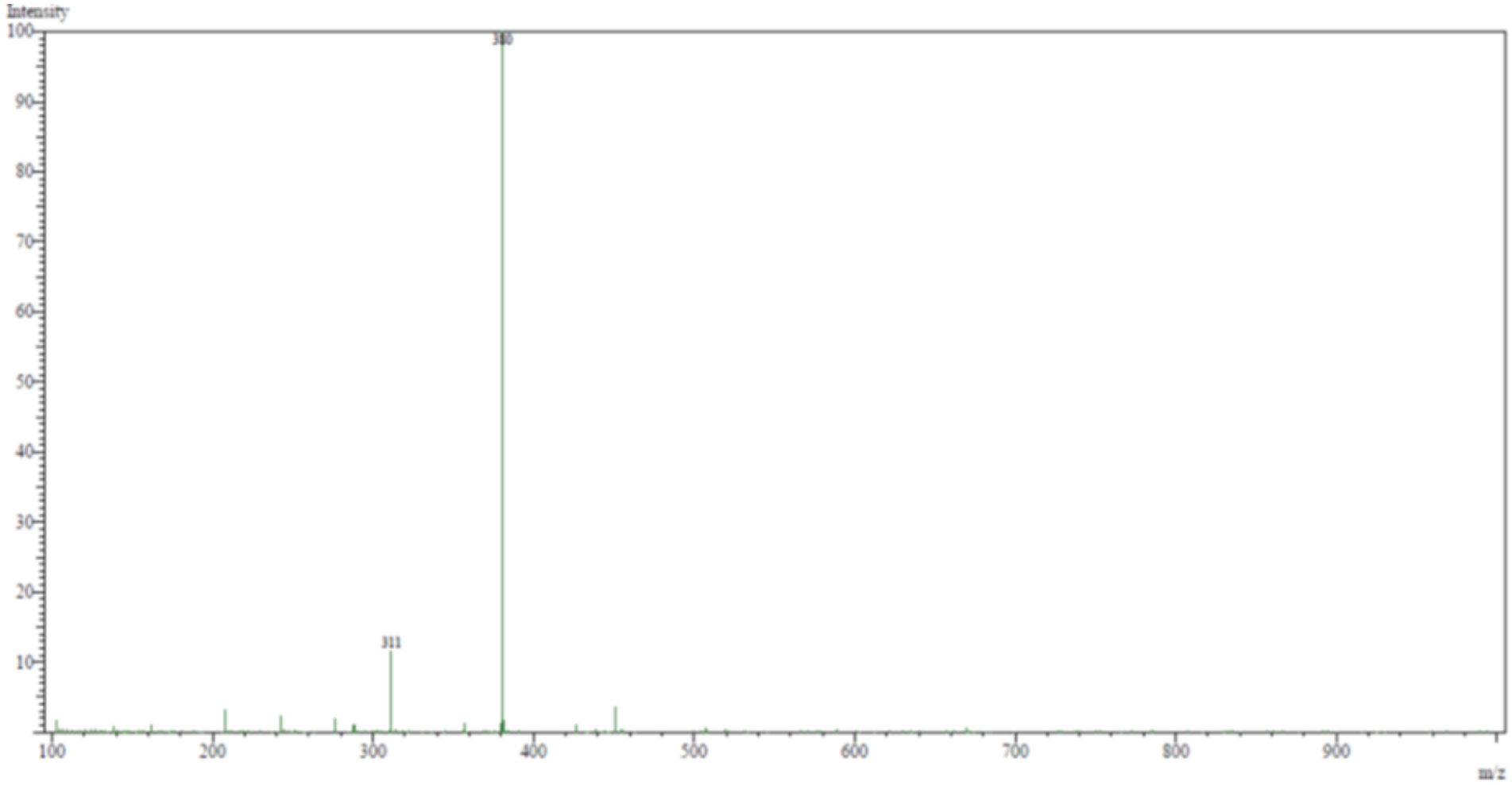

Figure 3.14: ESI-quadrupole mass spectrum acquired after 5 days for the reaction of 0.1 mM I_2 and 0.1 mM SCN^- in CH_3CN.

Job's plot, also called the continuous variation method was used to determine the stoichiometry of the I_2SCN^- complex. Stock solutions of equimolar concentrations (0.2 mM I_2 and 0.2 mM SCN^-) were used in these experiments. Each sample was prepared by mixing different volumes of I_2 and SCN^- such that the final volume remained constant and the mole fraction of I_2 ranges from 0 to 1. The absorbance of each solution when plotted against the mole fraction of I_2 resulted in Figure 3.15. The plot shows that the maximum is reached for X_{iodine} ~ 0.67 consistent with a 1:2 reaction stoichiometry for the I_2SCN^- complex.

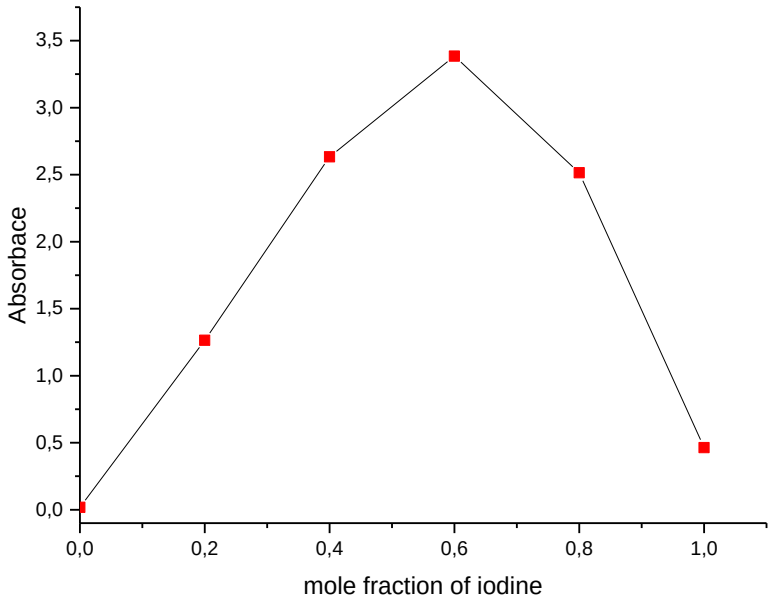

Figure 3.15: Job's plot for the I_2SCN^- complex at 305 nm.

3.2 Antibacterial activity testing

Several procedures to test antimicrobial activity have been reported in literature. One of the classic methods commonly used is the agar diffusion assay; where the antimicrobial species is positioned on the surface of the agar plate that has been inoculated with the test bacteria. During the time of incubation, the antimicrobial diffuses, causing a concentration gradient which manifest as the inhibition zone for bacterial growth.[76, 77] Automated systems of the classical procedure were developed in the early 1970s for antimicrobial susceptibility testing (AST).[78, 79]

3.2.1 Determination of antimicrobial activity by the Disk Diffusion Method

In this study, the antimicrobial activity of I_2 + SCN^- solutions in acetonitrile against the strains of *E. coli* and *E. faecalis*, namely *E. coli* ATCC 29522, *E. coli* FP 5, *E. coli* FP 29, *E. faecalis* ATCC 29212, *E. faecalis* GCE 8 and *E. faecalis* GCE 22 was determined by means of the disc diffusion method. Each experiment was performed in triplicate. The inhibition zones were measured with a calliper (Figure 3.16) and a clear zone indicates the absence of microbial growth and provides an indication of antimicrobial activity. Under the conditions of our experiments, mixtures of I_2 with SCN^- produced a yellow I_2SCN^- complex which has antimicrobial properties. Three mixtures including 0.05 mM (SCN^-) + 0.5 mM (I_2), 0.5 mM (SCN^-) + 0.5 mM (I_2) and 5 mM (SCN^-) + 0.5 mM (I_2) were tested. The diameters of growth inhibition in millimetres produced by the three solutions are presented in Table 3.1 as mean $\pm$ SD.

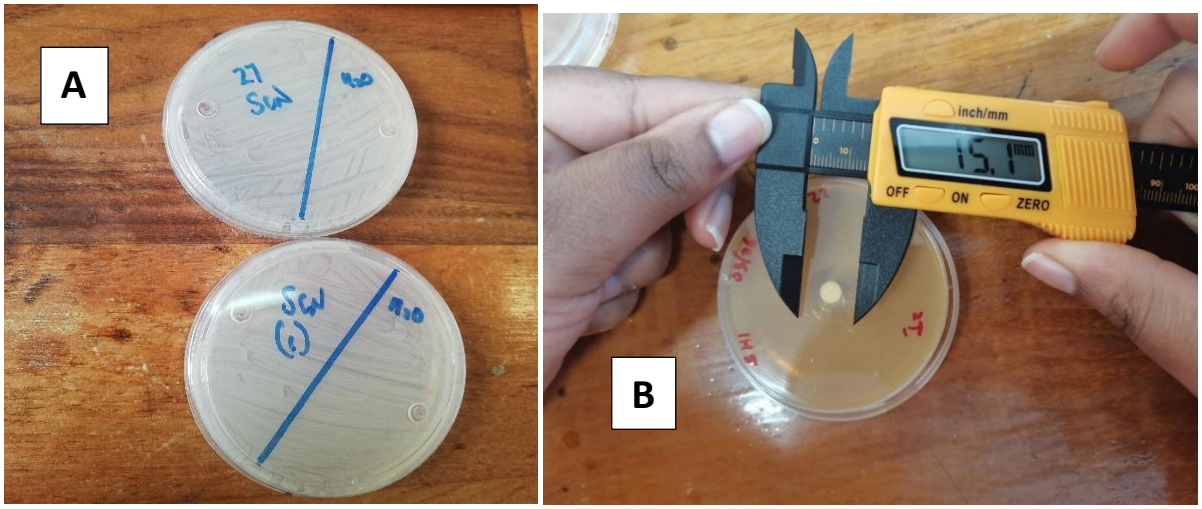

Figure 3.16: Images of petri-dish samples showing a zone of inhibition of I_2 [B] and an absence of a zone of inhibition for SCN^- [A].

When comparing the vulnerability of the different strains of *E. coli* and *E. faecalis* to the three mixtures, significant differences were observed (Table 3.1). *E. coli* proved to be more susceptible to the testing solutions in comparison to *E. faecalis*. This was proven as a larger zone of inhibition was observed for *E. coli* (Gram-negative bacteria) in comparison to *E. faecalis* (Gram-positive bacteria). When comparing the three solutions against the same strains, 0.5 mM (SCN^-) + 0.5 mM (I_2), had the strongest effect, followed by 0.05 mM (SCN^-) + 0.5 mM (I_2) (excess iodine) and then 5 mM (SCN^-) + 0.5 mM (I_2) (excess thiocyanate). No significant difference was observed on the inhibitory effect of the three solutions on all the investigated bacterial strains. However, all three solutions were less effective against *E. faecalis* ATCC 29212 (ZOI = 22.18 – 23.88 mm), *E. faecalis* GCE 8 (ZOI = 23.96 – 25.54 mm) and *E. faecalis* GCE 22 (ZOI = 18.17 – 26.30 mm) compared to *E. coli* FP 5 (ZOI = 34.51 – 39.12 mm), *E. coli* FP 29 (ZOI = 33.69 – 38.56 mm) and *E. coli* ATCC 29212 (ZOI = 33.70 – 37.28 mm).

The observation that excess thiocyanate lowers the antimicrobial activity of I^-/I_2 are in agreement with other literature reports.[80-82] The lowering of the activity by SCN^- was also observed in lactoperoxidase catalysed systems and was attributed to the binding competition between SCN^- and I^- on the LPO.[80, 82, 83] Furthermore, the results obtained are in line with the observation by Bosch *et. al*., where they observed strong antimicrobial effect against *Candida albicans, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa* by the lactoperoxidase system involving both the I^- and SCN^-.[24]

Table 3.1: Zones of inhibition induced by I_2SCN^- solutions after 24 hours of inoculation at 37 °C**.**

		Diameter of the zone diffusion disk averaged from triplicates disks (mm)				
Sample Name	**Variety of micro-organism**	**Blank** (Acetonitrile)	**Iodine**	**Excess Iodine** (0.5 mM I_2+ 0.05 mM SCN^-)	**Excess Thiocyanate** (0.5 mM I_2 + 5 mM SCN^-)	**Equimolar** (0.5 mM I_2 + 0.5 mM SCN^-)
E. coli ATCC 29522	Gram (-)	12.12 ± 0.01	20.15 ± 0.02	35.41 ± 0.00	33.69 ± 0.13	38.56 ± 0.02
E. coli FP 5	Gram (-)	13.05 ± 0.02	22.52 ± 0.00	38.56 ± 0.08	34.51 ± 0.01	39.12 ± 0.03
E. coli FP 29	Gram (-)	12.51 ± 0.01	19.98 ± 0.01	37.28 ± 0.01	33.70 ± 0.21	37.28 ± 0.01
E. faecalis ATCC 29212	Gram (+)	9.14 ± 0.03	14.71 ± 0.08	22.41 ± 0.03	22.18 ± 0.02	23.88 ± 0.00
E. faecalis GCE 8	Gram (+)	8.35± 0.03	14.22 ± 0.22	25.54 ± 0.02	23.96 ± 0.02	25.89 ± 0.02
E. faecalis GCE 22	Gram (+)	8.34 ± 0.02	15.0 ± 0.04	24.12 ± 0.02	18.17 ± 0.00	26.30 ± 0.01

The equimolar SCN^-/I_2 and excess SCN^-/I_2 solutions are more potent at killing bacteria compared to the sole iodine or thiocyanate solution. The oxidant diiodothiocyanate was more effective in killing bacteria in comparison to iodine's activity against the *E coli* and *E faecalis* isolates. However, it can be observed on Table 1 that increasing the concentration of SCN^- lowers the inhibition efficiency of the complex, I_2SCN^-. This is clear on *E. coli* ATCC 29522 where the I_2SCN^- complex's solution had excess SCN^- over I_2, the ZOI was 33.69 ± 0.13 mm which was the lowest in comparison to when we have an equimolar and excess I_2 solution which are 38.56 ± 0.02 mm and 35.41 ± 0.00 mm respectively. The complex creates a clearer zone of inhibition in a solution with 0.5 mM SCN^- in comparison to 5 mM of SCN^- which is the same trend for all the isolates.

I_2SCN^- prepared from acetonitrile significantly inhibited the growth of all the strains of Gram-positive bacteria and Gram-negative bacteria. I_2SCN^- containing equimolar solutions of SCN^- and I_2 demonstrated the most potent activity against all organisms tested with broad-spectrum activity while the I_2SCN^- containing excess SCN^- over I_2 demonstrated the least activity against both Gram-positive and Gram-negative bacteria on all the isolates. Gram negative FP 5 was more affected by the I_2SCN^- for the equimolar solution mixture, where the inhibition zone was found to be 39.12 ± 0.03 mm. The Gram-positive GCE 22 was more affected by the I_2SCN^- for the equimolar solution mixture, the inhibition zone was 26.30 ± 0.01 mm. Gram negative bacteria ATCC 29522 was less affected by the I_2SCN^- for the excess SCN^- solution mixture, and the inhibition zone was 33.69 ± 0.13 mm, while the Gram-positive GCE 22 was the least affected by the I_2SCN^- for the excess SCN^- solution mixture, the inhibition zone was 18.17 ± 0.00 mm.

All three ratios displayed a level of inhibition in all the isolates of Gram-positive and Gram-negative bacteria. However, I_2SCN^- compound with a mixture of iodine with an excess of thiocyanate resulted in minimal inhibition in comparison with others. Similar results have been reported which shows that an additional thiocyanate lowers the antimicrobial activity of I^-/I_2.[80-82] The lowering of the activity of iodine based antimicrobial agents with SCN^- was also observed in an enzyme catalysed reaction for the formation of ICN using LPO-H_2O_2-SCN^-/I^- system which was caused by the binding competition between SCN^- and I^- on the LPO.[80, 82, 83] However, these experiments were performed in the absence of LPO, therefore this observation can be linked

to the higher influence of thiocyanate-mediated bacteriostatic effect with an increasing concentration of thiocyanate.[84]

The disadvantage in the utilization of agar diffusion method to establish if a compound possesses an antimicrobial activity is that the antimicrobial effect may be indirectly influenced by the concentration of the compound or salt used, agar type, molecular size of the antimicrobial component, and incubation temperature.[85, 86] The antimicrobial activity on all tested ratios of I_2: SCN^- exhibited good antimicrobial activities at different dose levels of the prepared I_2SCN^- in the case of the gram-positive and gram-negative strains by showing clear zones of inhibition which were stable for up to 30 days.

3.2.2 Growth kinetics of *E. coli* and *E. faecalis* isolates

A quantitative evaluation of the antibacterial activity of I_2SCN^- at various concentrations against *E. coli* and *E. faecalis* strains was performed by measuring the optical density. Optical density at 600 nm (OD_{600}) is a commonly used method in biological studies to quantify the bacteria after treatment with an antimicrobial substance by measuring the absorbance at 600 nm .[87, 88] In our experiments, growth was monitored every 30 minutes for 6 hours and the last reading was recorded after 24 hours of incubation. In a previous study by Bafort *et. al*. enhanced antimicrobial activity against *P. expansum* by lactoperoxidase generated I_2SCN^- at concentrations between 0.15 – 0.45 mM was reported.[20] In this study, I_2SCN^- concentrations of 0.10, 0.25 and 0.50 mM were used for both *E. coli* and *E. faecalis* strains. Appropriate controls with *E. Coli* or *E. faecalis* were included for the efficacy evaluation of I_2SCN^-. The growth curves were plotted as optical density at 600 nm (OD_{600}) on the y-axis against time on the x-axis. As can be seen from the curves, an increase in I_2SCN^- concentration enhanced antibacterial activity for both strains of bacteria. Growth curves of *E. Coli* are shown in figures 3.17 - 3.20 and in the appendix section (figures A2 – A4) and those of *E. faecalis* are indicated in figures 3.21 - 3.24 and A5-A7 treated with I_2SCN^-.

The bacterial growth curves are generally sigmoidal and revealed three distinct phases, namely, the lag, exponential, and stationary growth phases. The lag phase is the adaptation phase in which the effect of I_2SCN^- on all the strains was not visible. Monod has described the lag phase as an incomprehensible growth phase which may be influenced by unspecified

regulatory mechanisms.[89] Generally, the growth curves showed extended lag phase for *E. coli* compared to *E. faecalis*. In both strains, the lag phase continued for 240 min after which the stationary phase started. This result confirms that the Gram-negative bacteria are more susceptible to I_2SCN^- compared to the Gram-positive bacteria. The reduced antibacterial activity against *E. faecalis* is expected since it has a more thicker cell wall which is harder to penetrate than *E coli*.[90]

The data in Figures 3.17 - 3.20, shows that an increase in I_2SCN^- concentration results in the reduction of cell viability by 13 – 25% (ATCC 29522), 5 – 23% (FP 5) and 19 – 29% (FP 29) after 5.5 hrs of incubation. The 98% antimicrobial efficacy observed by Bafort *et. al*. can be explained by the likelihood of the peroxidase system to generate a mixture of oxidation products including cyanogen iodide (ICN) when both I^- and SCN^- are present. Cyanogen iodide is an oxidant that can kill *E. coli* efficiently at very small concentrations < 1 μM.[84] In contrast, I_2SCN^- was found to be less effective, suggesting that it is rather a milder oxidant compared to ICN.

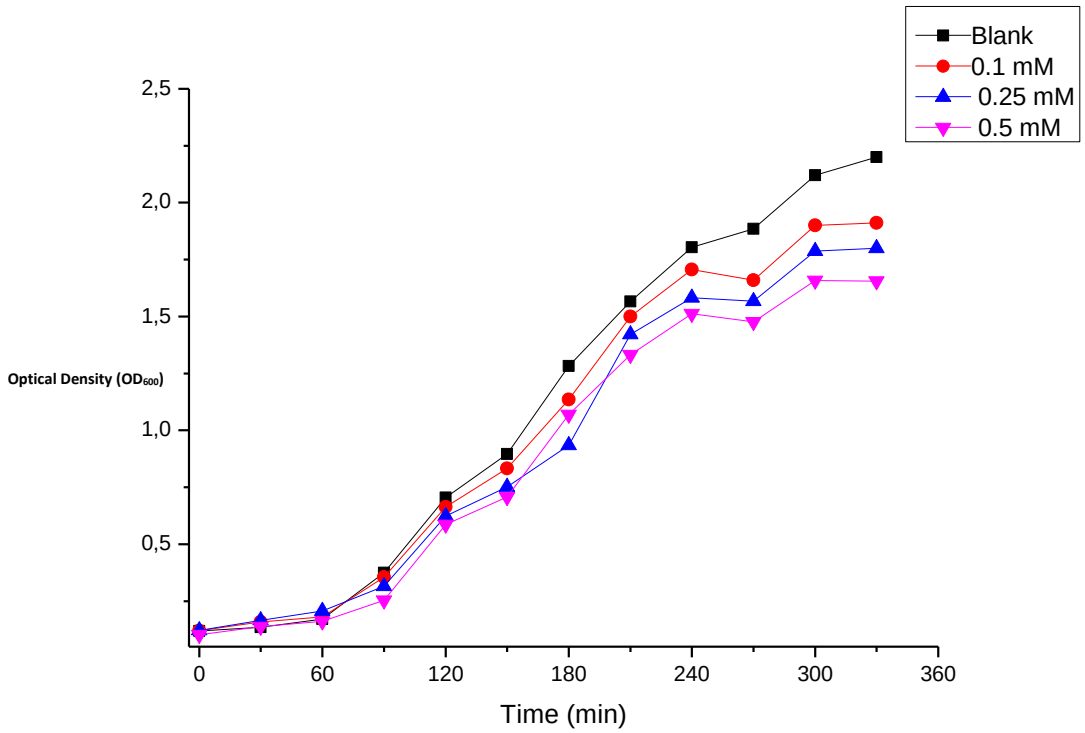

Figure 3.17: Growth kinetic curve for *E. coli* ATCC 25922 with varying I_2SCN concentration over 5.5 hours in NA liquid media at 37 °C.

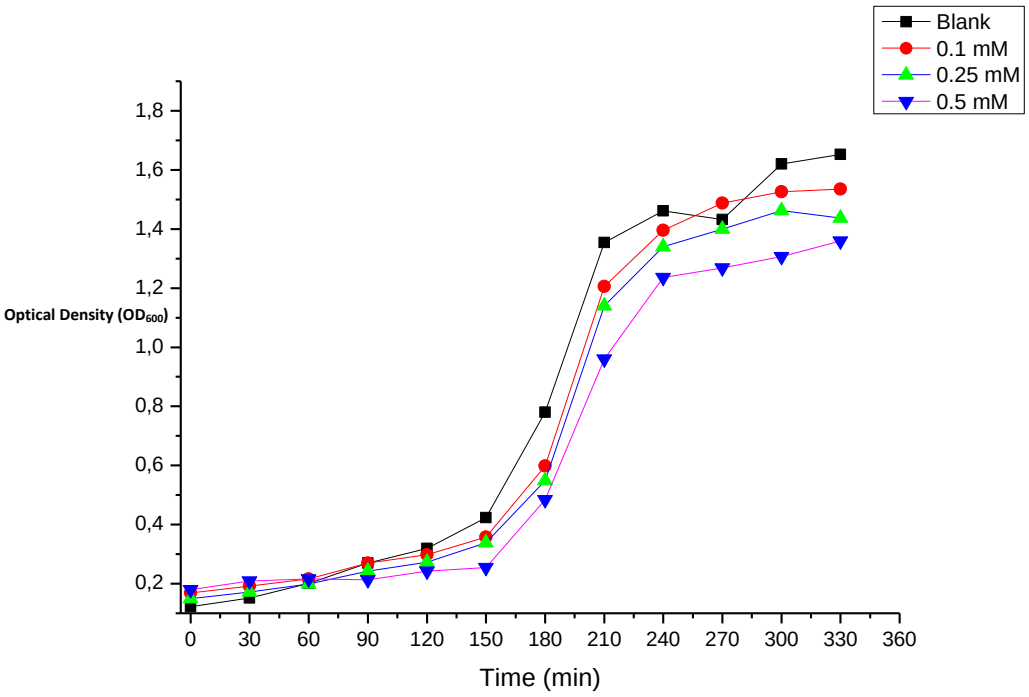

Figure 3.18: Growth kinetic curve for *E. coli* ATCC FP 5 with varying I_2SCN concentration over 5.5 hours in NA liquid media at 37 °C.

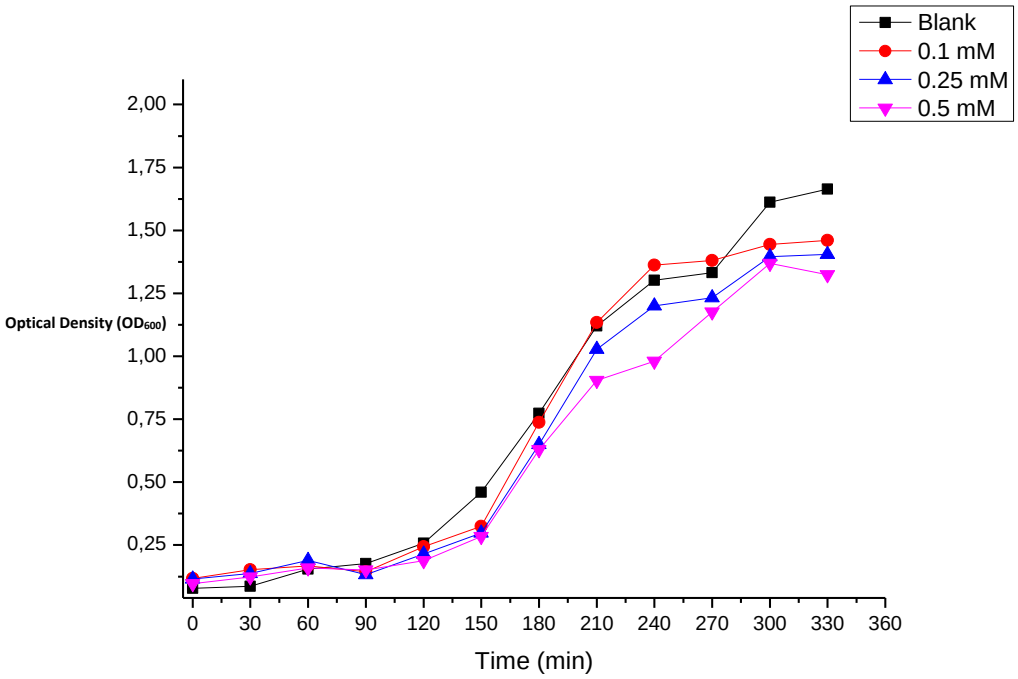

Figure 3.19: Growth kinetic curve for *E. coli* ATCC FP 29 with varying I_2SCN concentration over 5.5 hours in NA liquid media at 37 °C.

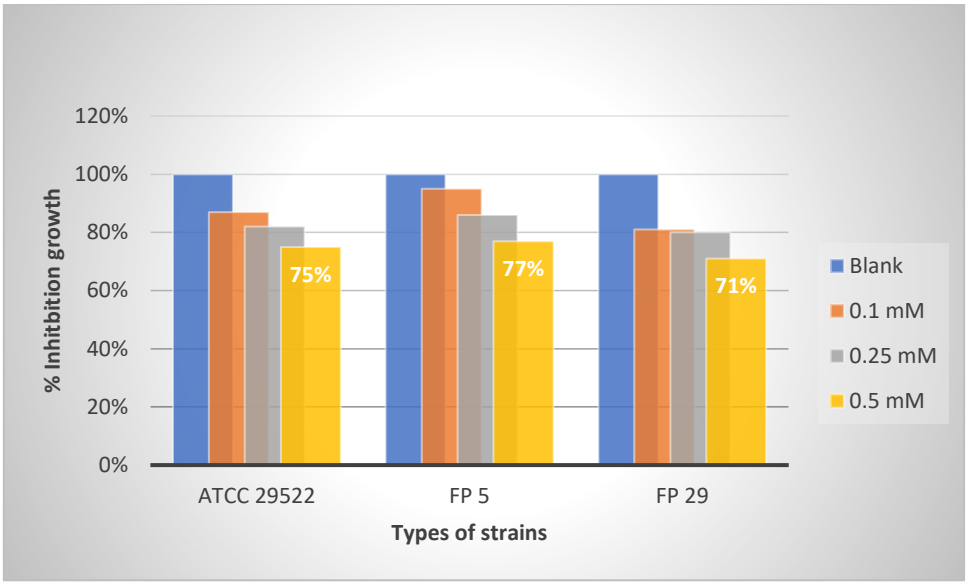

Figure 3.20: The inhibition of the growth of three isolates of the *E. coli* (ATCC 29522, FP 5, FP 29) over 5.5 hours in NA liquid media at 37 °C.

I_2SCN^- was less effective in killing *E. faecalis* (Figures 3.21 – 3.24) as cell viability was only reduced by 5 – 12% (ATCC 29522), 3 – 8% (GCE 8) and 5 – 13% (GCE 22) after 6 hrs incubation. The results indicate a small concentration-dependence in killing of *E. faecalis*.

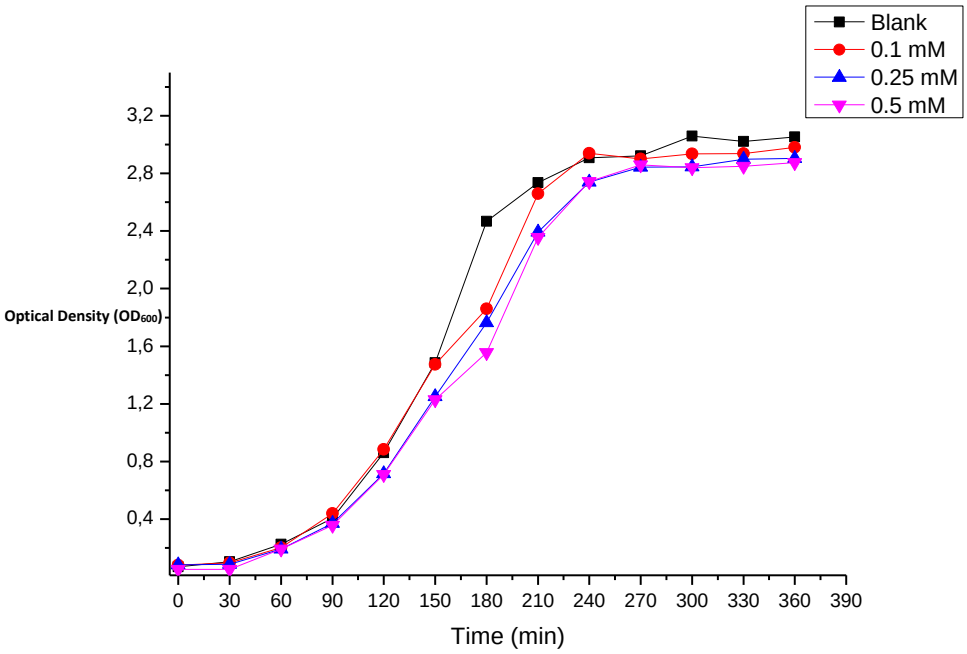

Figure 3.21: Growth kinetic curve for *E. faecalis* ATCC 29212 with varying I_2SCN concentration over 6 hours in BHI liquid media at 37 °C.

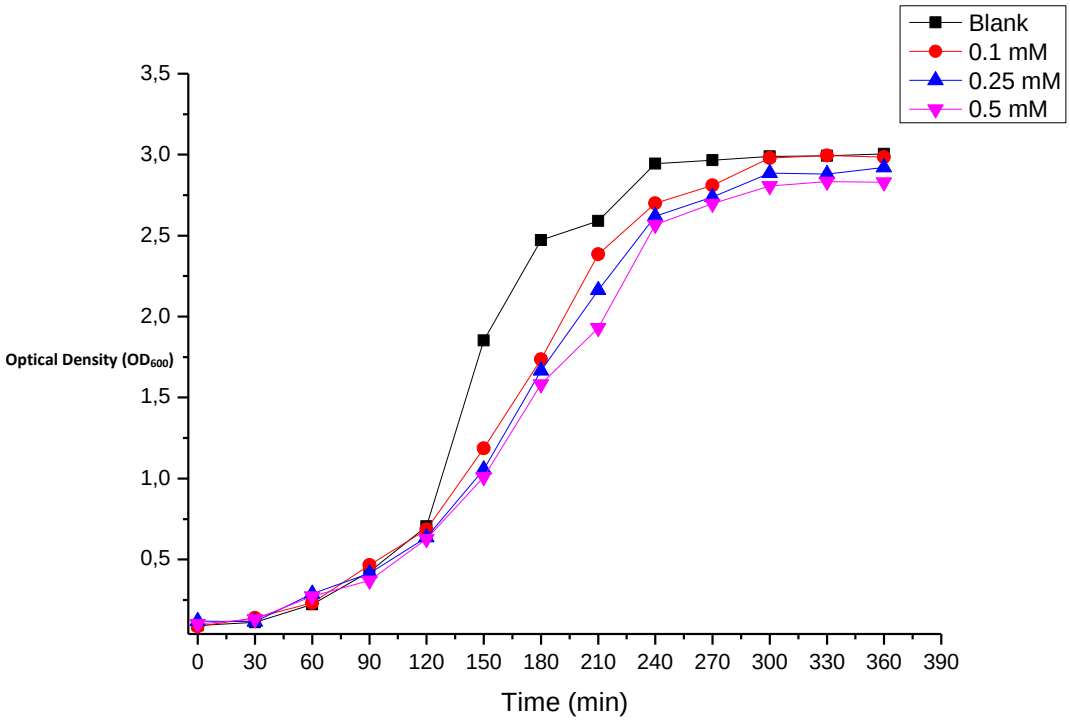

Figure 3.22: Growth kinetic curve for *E. faecalis* GCE 8 with varying I_2SCN concentration over 6 hours in BHI liquid media at 37 °C.

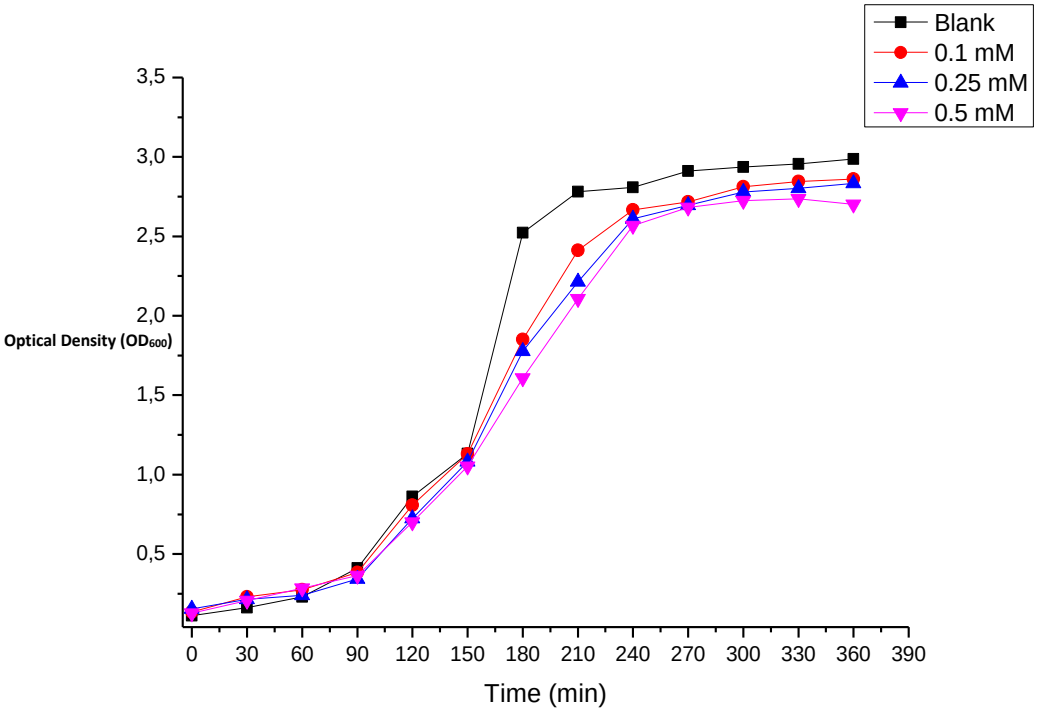

Figure 3. 23: Growth kinetic curve for *E. faecalis* GCE 22 with varying I_2SCN concentration over 6 hours in BHI liquid media at 37 °C.

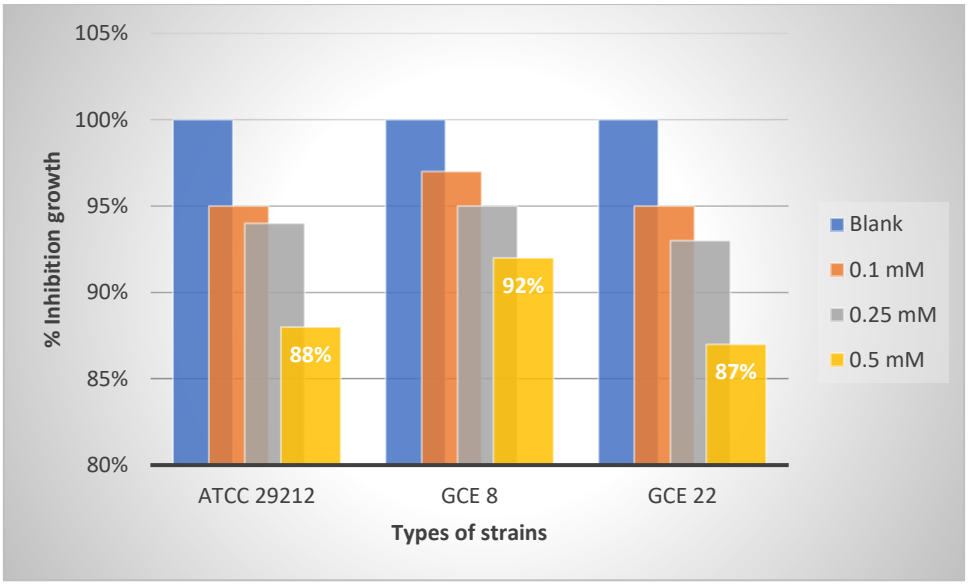

Figure 3.24: Inhibition effect of I_2SCN^- on the growth of the *E. faecalis* with varying I_2SCN concentration over 6 hours in BHI liquid media at 37 °C.

The antibacterial growth was monitored using 0.10 mM, 0.25 mM, and 0.50 mM concentration of I_2SCN^-. For ATCC strain, I_2SCN^- reduced the bacteria biomass by 5%, 6%, and 12%, respectively over 6 hours compared to the growth of the control labelled as a blank. When the concentration of I_2SCN^- is 0.10 mM, 0.25 mM and 0.50 mM, the bacteria biomass reduced by 3%, 5%, and 8%, respectively for GCE 8 strain in 6 hrs in comparison with the control. For GCE 22 biomass it was reduced by 5%, 7%, and 13% using 0.10 mM, 0.25 mM, and 0.50 mM of I_2SCN^- over 6 hours.

The bacterial growth curves revealed three different phases, namely, the lag, exponential, and the stationary growth phase. The lag phase is known as the adaptation phase, in this phase, the effect of I_2SCN^- on all the strains was not visible. Monod has reported the lag phase as an incomprehensible growth phase which may be influenced by unspecified regulatory mechanisms.[89] The lag phase allows the bacterial cells to adapt to new environmental conditions, this lasted longer in the *E. coli* compared to the *E. faecalis* strains.

The bacterial growth decrease is visible or starts to show on the exponential growth phase where the bacterial growth disturbance is observed. The strongest effect was observed at the stationary phase where there is no longer a population growth since the number of dividing cells are equal to the number of dying cells. Provided with more time, the pathogenic bacteria

get to produce virulence factors which assist them in surviving harsh conditions, thus leading to a cell/bacteria death. In this study the effect of bacterial growth was monitored for 24 hours, leading to the last observation of a stationary phase which shows that the increase in concentration, increases the effectiveness.

Bacterial growth in BHI liquid media is rapid since the media is rich in lipids, protein, and carbohydrates. Hence it is not surprising to have lower effectiveness of the compound in BHI liquid media growth since penetrating the bacterial cells by the I_2SCN^- will be more difficult in such media. The tolerance of the bacteria is higher compared to the zone of inhibition testing. I_2SCN^- was still effective as an antimicrobial in NA and BHI liquid media for both *E. coli* and *E. faecalis* strains. The lower effectiveness on *E. faecalis* strain is expected since they have a thicker cell wall in comparison to *E. coli* strain.

3.3 Biological significance of non-enzyme I_2SCN^- formation

All human defensive peroxidases which use hydrogen peroxide as an oxidant can utilize both iodide and thiocyanate as substrates to produce iodide-based antimicrobials. The new iodide-based antimicrobials are of great interest as they are potential alternatives that could solve the antimicrobial resistance problem described in literature. Schlorke *et. al*. (2016) showed that the LPO-H_2O_2-SCN^-/I^- system produces hypothiocyanite, reactive iodine species and cyanogen iodide. The LPO-H_2O_2-SCN^-/I^- system produces ICN under conditions of excess I^- over SCN^-. At a ratio of 1:10 to 1:4 for SCN^-/I^-, the LPO system showed high antimicrobial efficacy against *E coli*. When pure ICN was used, a concentration as low as 1 μM, caused an immediate biocidal effect on *E coli*.[67] In contrast, the results of Bafort et. al. (2018) showed that in acidic buffer solutions that has ratios of 1:4.5 for SCN^-/I^-, the LPO system produces a mixture of I_2SCN^- and $I(SCN^-)_2$. The LPO - 6.6 mM H_2O_2 - 1.2 mM KSCN/5.4 mM KI mixture exhibited antimicrobial activity at various dilutions against *P expansum*. In neutral tap water the LPO system did not produce any I_2SCN^- or $I(SCN^-)_2$ but formed HOI and $OSCN^-$.[20]

In addition to peroxidase-derived iodide-based antimicrobials, secondary products of iodide from non-enzymic reactions occurring after the production of hypohalites (including OCl^-, OBr^-, OI^- and $OSCN^-$) are likely the ones controlling microbial growth under physiological conditions. In the oral cavity, SCN^- is a major substrate (0.1 – 3 mM) and $OSCN^-$ is the dominant product of peroxidases in saliva. Hypothiocyanite (and other hypohalites) can then oxidize I^-

to I_2 followed by the reaction of I_2 with SCN^- to produce interhalogen chemical species such as ICN, $I(SCN)_2^-$ and I_2SCN^-. The most cytotoxic of these interhalogens is ICN which has a half-life of approximately 17 years at physiological pH conditions. The present work has shown that ICN can react with SCN^- to produce I_2SCN^-.

There are only a few studies in literature that have investigated antimicrobial activity of I_2SCN^- against human and plant pathogens. Several researchers have mainly focused on peroxidase and glucose oxidase catalysed systems involving I^- and SCN^- which produces a mixture of antimicrobial chemical species.[43, 67, 80, 91-93] Although the reaction of I_2 with SCN^- has been known for a while, contradictory results about its products including ICN, ISCN, $I(SCN)_2^-$ and I_2SCN^- have been reported.[19, 53, 94, 95]

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Chapter 4: Conclusion

4.1 General conclusion

In this study we have demonstrated that mixtures of iodine (I_2) with thiocyanate (SCN^-) in acidic water and in acetonitrile produces a product with antimicrobial properties. The intensely yellow complex resulting from this chemical reaction was identified as diiodothiocyanate (I_2SCN^-) by UV-Vis spectrophotometry and ESI-MS. Through these techniques, the products obtained at pH 3.6 in water showed two absorption bands at 300 nm and 350 nm and a signal at 311 (m/z). Under these experimental conditions, I_2SCN^- coexists with triiodide (I_3^-) which also absorbs at 300 and 350 nm and has a signal at 380 (m/z). The presence of I_2SCN^- was further confirmed by the reaction of thiocyanate (SCN^-) with cyanogen iodide (ICN) in water at pH 7.0. A yellow-coloured product exhibiting a strong absorption band at 300 nm, an intense signal at 49.4 ppm and a mass at 311 (m/z) was identified by UV-Vis spectrophotometer, ^{13}C NMR spectroscopy and ESI-MS respectively. In water, I_2SCN^- is short-lived but exhibits high stability in acetonitrile.

The antimicrobial activity of I_2SCN^- was tested against strains of *E coli* and *E faecalis* namely *E. coli* ATCC 29522, *E. coli* FP 5, *E. coli* FP 29, *E. faecalis* ATCC 29212, *E. faecalis* GCE 8 and *E. faecalis* GCE 22. We investigated three solutions prepared by mixing 0.5 mM I_2 with 0.05 mM, 0.5 mM and 5 mM SCN^-. The present work demonstrates that all three test solutions showed stronger antimicrobial activity against Gram-negative (*E. coli*) than Gram-positive (*E. faecalis*) strains of bacteria. This observation is not surprising given the differences in structural features between the two strains. The *E faecalis* strain has a thicker cell wall compared to the *E coli* strain which makes it difficult for an antibacterial compound to penetrate and kill the cell. When comparing the antimicrobial efficacy of these solutions against the same strains, the equimolar solution had the strongest effect whereas the solution containing excess SCN^- was the least effective.

4.2 Future work

The results of these experiments show that non-enzymic reactions of I_2 with SCN^- produce I_2SCN^- which has good antimicrobial potential. In comparison with ICN, a more potent antimicrobial produced by the human defensive peroxidase system, I_2SCN^- which was also formed by the reaction of ICN with SCN^- appears to be a mild antimicrobial. Future studies should investigate other non-enzyme pathways likely to result in the formation of I_2SCN^- in the human body. In addition, these studies should also investigate the formation of other iodine-based antimicrobials through non-enzyme pathways as alternatives to current antimicrobials. Furthermore, the antimicrobial activity of I_2SCN^- should be tested against plant pests for possible application in agriculture.

Chapter 5: Appendix

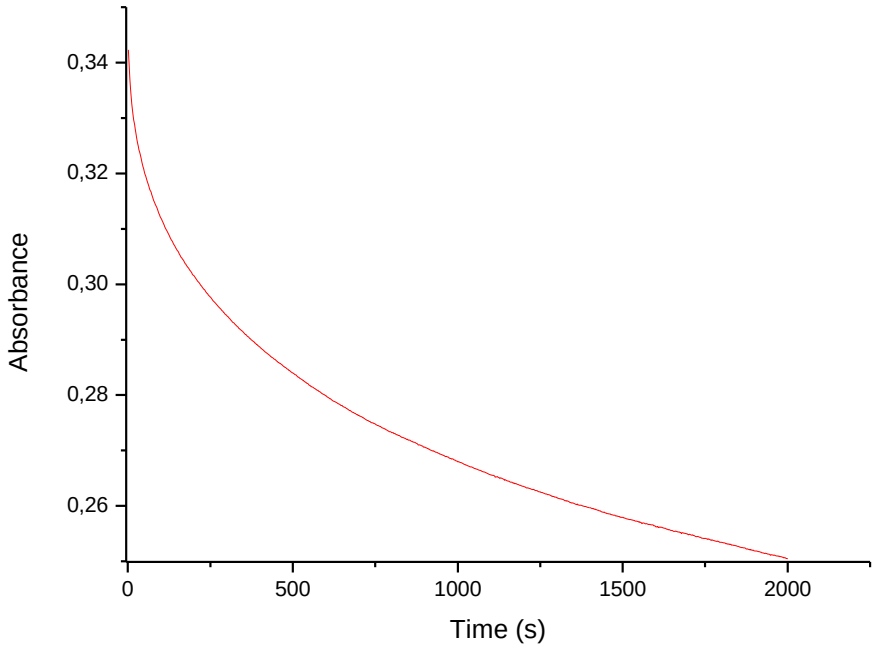

Figure A-1: Stopped flow kinetic trace of I_2 (0.1 mM) and SCN^- (0.1 mM) for the disappearance of I_2 at 460 nm (pH of 3.6).

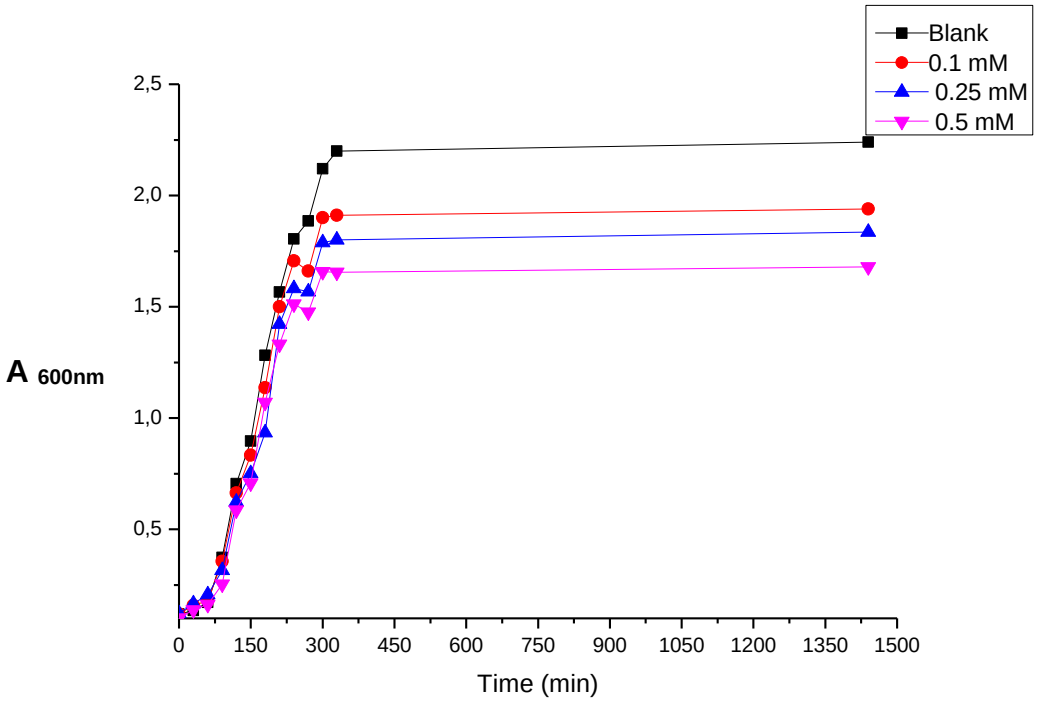

Figure A-2: Growth kinetic curve for *E. coli* ATCC 25922 with varying I_2SCN concentration over 24 hours in NA liquid media at 37 °C.

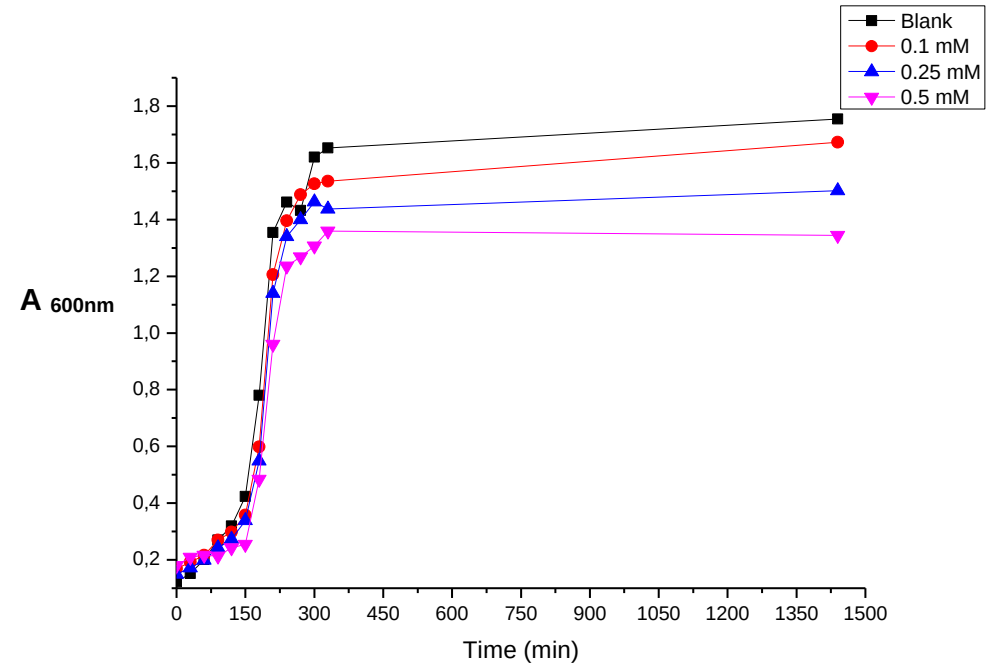

Figure A-3: Growth kinetic curve for *E. coli* ATCC FP 5 with varying I_2SCN concentration over 24 hours in NA liquid media at 37 °C.

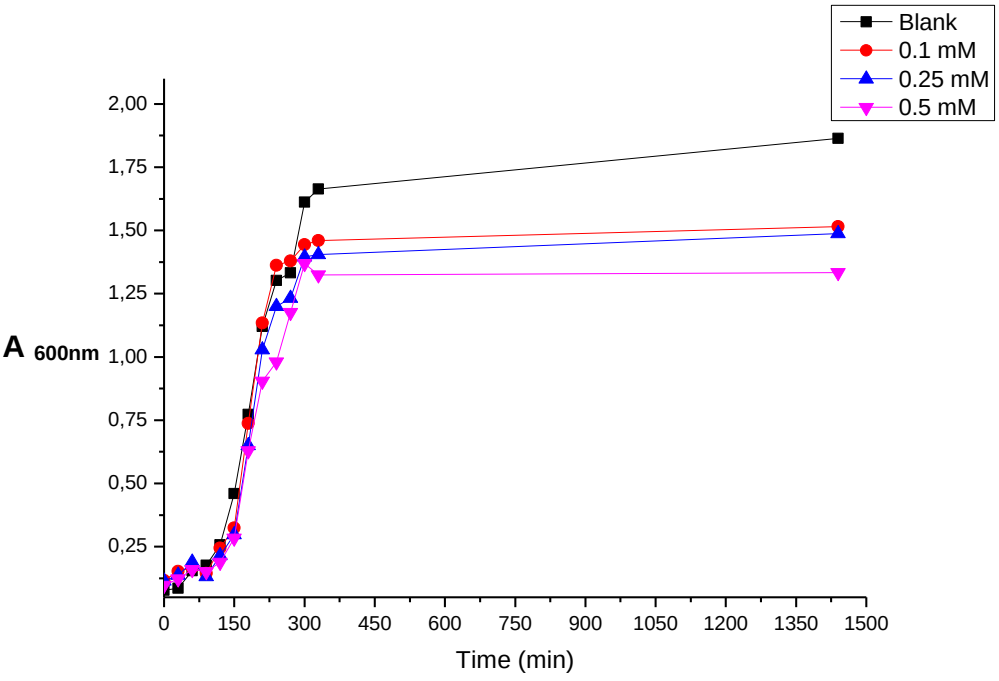

Figure A-4: Growth kinetic curve for *E. coli* ATCC FP 29 with varying I_2SCN concentration over 24 hours in NA liquid media at 37 °C.

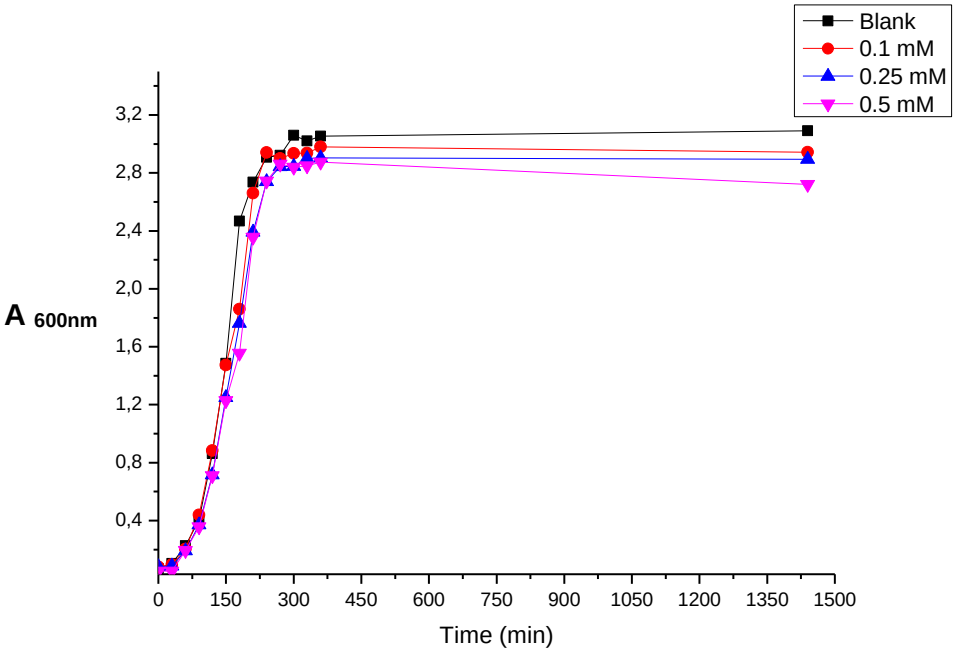

Figure A-5: Growth kinetic curve for *E. faecalis* ATCC 29212 with varying I_2SCN concentration over 24 hours in BHI liquid media at 37 °C.

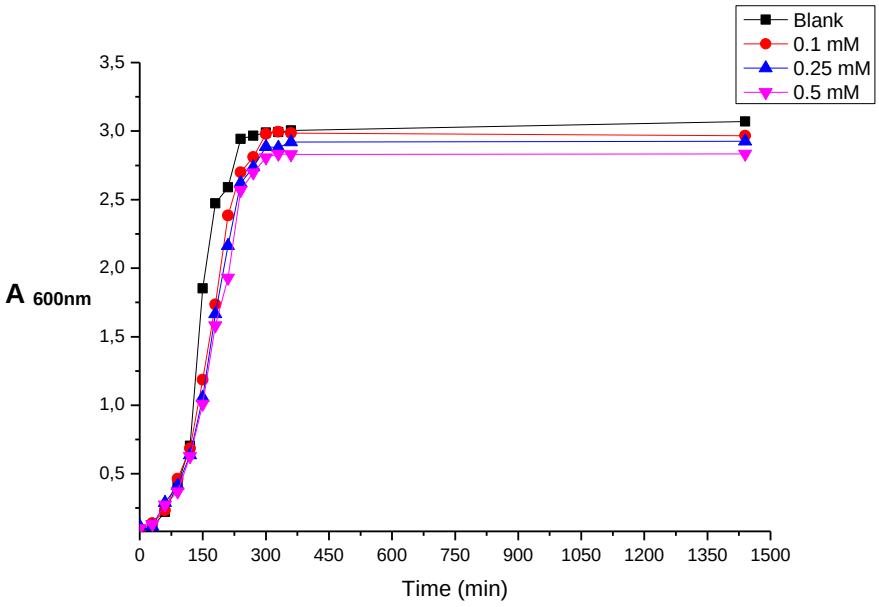

Figure A-6: Growth kinetic curve for *E. faecalis* GCE 8 with varying I_2SCN concentration over 24 hours in BHI liquid media at 37 °C.

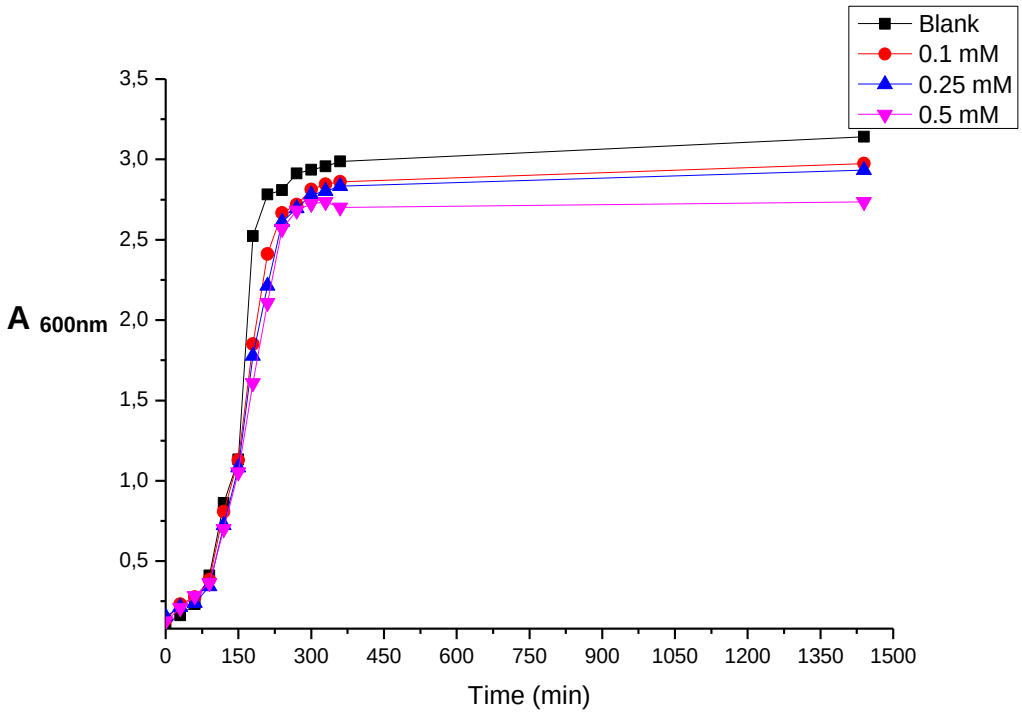

Figure A-7: Growth kinetic curve for *E. faecalis* GCE 22 with varying I_2SCN concentration over 24 hours in BHI liquid media at 37 °C.