Optimization of Chitinase Produced by Microorganisms Isolated from the Soil Degraded Crab Shell Waste

A. VIJAYALAKSHMI

(13PBC013)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore – 641043

In Partial Fulfilment of the Requirement for the Degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

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Signature of the Head of the Department

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Signature of the Supervisor

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LIST OF ABBREVATIONS

FAO	Food and Agriculture Organization
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
SSP	Shrimp Shell Powder
SCSP	Shrimp and Crab Shell Powder
SCP	Single Cell Protein
GlcNAC	N-acetyl-D-glucosamine
SSF	Solid State Fermentation
SmF	Submerged Fermentation
MALDI - TOF	Matrix Assisted Laser Desorption Ionization – Time of Flight
LT	Lethal Time
AMCase	Acidic Mammalian Chitinase
CSS	Crab Shell Soil
CSW	Crab Shell Waste
CCA	Colloidal Chitin Agar
CV - I	Crystal Violet – Iodine
MR – VP	Methyl Red – Voges Proskauer
TSI	Triple Sugar Iron
BSA	Bovine Serum Albumin

Introduction

1.0 INTRODUCTION

In recent years, the exhaustion of marine resources is a reality all over the world even in the case of resources regulated internationally by Food and Agriculture Organization (FAO) through the Regional Fishery Bodies. Annual discard of the marine wastes are estimated to be 20 million tons worldwide, which indicates 25% of the catch and include non-targeted species, processing waste and by-products. Waste and by-products discharged by the fisheries are currently increasing and it possess a serious environmental problem (Ferraro *et al*., 2010).

The increase of shrimp and crab production also increases the amount of waste, which, if not handled seriously, will cause environment pollution. That noted 55 – 60% out of the total crab production consists of waste/garbage, which are in the forms of crab shell. If disposal of crab shell are neglected, it will cause an increase of Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) and as a consequence, causes environmental pollution (Darmanto, 2002).

The seafood consumption has consistently increased during the recent years, as seafood has been progressively recognized as an abundant source of nutrients for human health. A very high percentage of captures are not considered useful because they are not profitable due to their low consumer acceptability. Nevertheless, these captures apart from reducing waste of marine resources, may have intrinsic nutritious value as a source of high value added compounds, suitable for human health applications (Lage - Yusty *et al*., 2011).

Marine invertebrates are widely being used as food and feed supplement around the world and may be used in enrichment of diet and new value-added products to decrease processing waste and increase profits. These by-products have applications in the food industry, including health-

promoting ingredients like marine oils and calcium, as well as enzymes, antioxidants, flavorings and pigments. Crabs, among many other invertebrates, are considered to be an important shell fishery product. The demand for products derived from fishing has increased considerably (Shahidi, 2006).

Crustacean-processing waste or by-product has traditionally been added to animal feeds. These by-products contain pigments, flavor components, calcium and other minerals, as well as high-quality protein and can also be used to create value-added products. Marine by-products indicate a source of lipids of interest to the health, nutrition, aquaculture and cosmetology industry (Lage - Yusty *et al*., 2011).

Crab exploitation produce important by-products because of their abundance and relative ease of capture. These shell wastes constitute valuable sources of components and a potential new industrial resource, because it is a nutritional and valuable source of protein, lipids and carotenoids (Beaulieu *et al*., 2009).

Crab processing bio-wastes include the head, shell and tail portions (non-edible parts) which account for about 50–70 % of the total volume of raw materials. The global implications of this scenario are that enormous shrimp bio-wastes are being generated by the seafood industry due to the escalating demand for crab products. In addition, continuous disposal of these voluminous bio-wastes into coastal and near shore environments has contributed to intense environmental pollution and consequent deterioration in affected ecosystems.

On the other hand, by disposing the valuable by-products without recycling and proper utilization, the sea food industries are missing prime opportunities for deriving several value added products such as chitin and other bioactive substances (Bhaskar *et al*., 2010).

Enormous amounts of chitin can be found in the biosphere; it is the major component of cuticles of insects, fungal cell walls, yeast or green algae. Fungi provides the largest amount of chitin in the soil (6–12 % of chitin

biomass, which is in the range of 500–5000 kg). Chitin is also widely present in crab and shrimp shells. A working estimate for the annual turnover is in the range of 1010–1011 tonnes, making chitin as one of the most abundant biopolymers. Chitin can be readily obtained by simple extraction. To date, the major source of industrial chitin comes from wastes of marine food production, mainly crustacean shells, *e.g.* shrimp, crab or krill shells (Arbia *et al*., 2013).

In the processing of shrimps for human consumption, between 40 and 50 % of the total mass is waste. About 40% of the waste is chitin, incrusted with calcium carbonate and astaxanthin and containing meat and a small amount of lipid residues. A small part of the waste is dried and used as chicken feed, while the rest is dumped into the sea, which is one of the main pollutants in coastal areas. The utilization of shellfish waste has been proposed not only to solve environmental problems, but as a waste treatment alternative to the disposal of shellfish wastes. Crustacean shell waste consists mainly of 30–40 % protein, 30–50 % calcium carbonate and 20–30 % chitin, with species and seasonal variations (Kurita *et al*., 2006).

Marine microorganisms have recently emerged as a novel source for the isolation of industrial enzymes (Subramani and Narayanasamy, 2009). Whereas, marine environment, which encompass about 71% of the earth's surface and a vast resource for useful enzymes, remain unexplored. Marine microorganisms take active part in the mineralization of complex organic matter through degradative pathways of their metabolism in the marine environments and contribute to the secondary production in sea. Microorganisms as the fastest means to search for food may often use cell – free extracellular enzyme.

Bacteria and fungi from marine environments secrete different enzymes based on their habitat and their ecological functions. Marine microbial enzymes have become the focal points of interest and several enzymes have drawn the attention of the microbial prospectors and a few enzymes were isolated from sea water and marine sediments purified and characterized for their properties and applications (Das *et al*., 2012b).

In general, traditional microbial enzyme technology ventures are undergoing rapid transformation through the process of evolution of innovative technologies facilitated through techniques like molecular engineering and immobilization of enzymes (Shanmugaiah *et al*., 2008).

Chitinase are a group of enzymes that decompose chitin. It is the second most abundant polymer in nature and the most abundant in the marine environment. Chitinase break down chitin into a variety of products that include the deacylated oligomer chitosan and the disaccharide chitobiose are increasingly finding use in diverse fields, such as biomedicine, agriculture and even in cosmetics. The enzymes themselves have been found to be antifungal and nematicidal agents for agricultural biocontrol methods. The seafood industry is a major source of chitinous wastes. The recycling of which is extremely important to retain the carbon nitrogen balance in the ecosystem. Chitinases are considered to have the potential for use in the management of these wastes through the process of composting (Bansode and Bajekal, 2006).

Moreover, chitinases can be used for the control of pathogenic fungi in agriculture and the degradation of crustacean chitinous waste in sea food industry. These enzymes are also useful for the preparation of single-cell protein, the isolation of protoplasts from fungi and yeast and so on. Microbial chitinase has been produced by liquid fermentation processes (batch, continuous and fed-batch fermentation) and is commercially available at a high cost. Generally, the production is controlled by physical factors (aeration, pH and incubation temperature) and by the growth media components (Dahiya *et al*., 2006).

In order to increase the supply of active chitinase, it is necessary to reduce the production cost by using wastes for microbial growth. In this perspective, various chitinous materials from marine sources [shrimp shell powder (SSP), shrimp and crab shell powder (SCSP)] have been utilized for chitinolytic enzyme production as alternative to waste disposal. Interestingly, using shellfish chitin-containing wastes as the sole carbon/nitrogen source for

chitinase producing strains can be exploited as potential biocontrol agents (Rebah and Miled, 2013).

Since there is a need for the production of large quantities of this enzyme from different microbial sources due to its ever increasing demand in the industrial sector. The necessity to enhance their production is of considerable interest.

Owing to the potential biotechnological applications of chitinase, the bioprospectivity of chitinase producing microbes from different environment conditions is the need of the hour. Hence the present project entitled "**Optimization of Chitinase Produced by Microorganisms Isolated from the Soil Degraded Crab Shell Waste**" was carried out with the following objectives:

- ✓ To isolate chitinase producing microbes from decomposed crab shell and the soil samples collected from crab shell disposal site in Coimbatore.
- ✓ To identify the chitinase producing microorganisms.
- ✓ To optimize the chitinase activity of the microorganisms using different optimization conditions.

Review of Literature

2.0 REVIEW OF LITERATURE

In India, a major producer of crab, more than 1,00,000 tons of crab bio-waste are generated annually and only an insignificant amount of that bio-waste is utilized for the extraction of chitin while the rest is discarded or underutilized (Suresh and Kumar, 2012). Therefore, effective utilization of crab bio-waste has an important role not only in the maintenance of a clean environment but also in enhanced economic value through valorization of the same towards deriving a range of economically viable biomolecules (Bhaskar *et al*., 2010).

Bioconversion of chitinous materials into value added products has been proposed as a waste treatment alternative for the disposal and efficient management of shrimp bio-wastes and it is well accepted as the most effective and ecofriendly strategy for their utilization (Annamalai *et al*., 2011).

The review of literature pertaining to the present study entitled **"Optimization of Chitinase Produced by Microorganisms Isolated from the Soil Degraded Crab Shell Waste"** is discussed under the following headings:

2.1 Microbial Enzyme

2.2 Chitin

2.3 Chitinase

2.3.1 Types of Chitinase

2.3.2 Families of Chitinase

2.3.2.1 Bacterial Chitinase

2.3.2.2 Fungal Chitinase

2.3.2.3 Plant Chitinase

2.1 Microbial Enzyme

Microorganisms are the major source for the enzyme production. These enzymes are the catalytic milestones of metabolic activities of living being and catalyze most of the reactions in living organisms. Enzymes are proteins and possess properties such as specificity towards the reactions they catalyze and the substrates on which they act upon. Apart from basic approach, enzymes are widely studied in various aspects. From the industrial point of view, only a minimum number of enzymes are commercially available and few of them found to have applications in maximum quantities (Rao *et al*., 2006).

2.2 Chitin

Chitin, a linear and heteropolymer of β-1,4-N-acetylglucosamine (GlcNAC), is the second most important biopolymer on the earth. It is found in the outer skeleton of insects, fungi, yeasts, algae, crabs, shrimps, lobsters and in the internal structures of other invertebrates. The overall weight of shellfish (e.g. crab, krill and shrimp), approximately 75% of which is disposed as waste and chitin consists of 20-58% of that dry weight. Chitin has its use in order to boost up the production of extracellular chitinase. Chitin can be degraded by chitinase. The catabolism of chitin takes place in 2 steps, chitinases initially cleaves the chitin polymer into chitin oligosaccharides and further cleaves to N-acetylglucosamine and chitobiases another enzyme cleaves that N–acetylglucosamine into monosaccharides (Ohno *et al*., 2011).

2.3 Chitinase

Chitinases (E.C.3.2.1.14) are glycosyl hydrolases which catalyze the hydrolysis of insoluble chitin to soluble derivatives. They are produced by various groups of bacteria and fungi, and their physiological and ecological role vary with the source organism. Nevertheless, the production of microbial extracellular chitinases has received much attention recently due to their potential applications in the reclamation of seafood processing crustacean bio-waste, production of bio-active N-acetyl chitooligosaccharides (Suresh *et al*., 2011).

In spite of such industrial, environmental and biological significance, the high cost of the enzyme together with low activity and stability of available purified chitinases restrict its commercial exploitation (Suresh and Kumar, 2012). Hence, enormous efforts are being invested by investigators in harnessing new species of microorganisms, as well as different bioprocesses for economic enzyme production. Solid state fermentation (SSF) has been reported as a better alternative over conventional submerged fermentation (SmF) for cost effective production of microbial chitinases, since it can be carried out using cheap and readily available agro-industrial residues such as crab bio-waste (Suresh, 2012).

2.3.1 Types of Chitinase

Chitinase is an enzyme which hydrolyzes chitin into N-acetylglucosamine by either endocleavage or exocleavage of the β-1,3 and 1,4 bond. The enzyme is classified into several categories that can be based on their isolation, structural and functional characteristics (Sharma *et al*., 2011).

Chitinase can be split into two classes – one as exochitinases, which demonstrates the activity only for the non-reducing sugar end of the chitin chain and another class as endochitinases, which hydrolyse the internal β-1,4-glycosidic bonds on the chitin compound. Many endochitinases especially those with a high isoelectric point, exhibit an additional enzyme like lysozyme or lysozyme-like activity. Two isoforms of chitinase have been reported from several sources acidic chitinase and basic chitinase. Both the isoforms of chitinase are induced in response to pathogen attack and other environmental stimuli on the sources (Kirubakaran and Sakthivel, 2007).

2.3.2 Families of Chitinase

Chitinase is a large and diverse group of enzyme that shows differences in their molecular structure, substrate specificity and catalytic mechanism (CAZy, 2013).

Based on amino acid sequence similarity, chitinolytic enzymes are divided into families 18, 19 and 20 of glycosyl hydrolases. Family 18 is diverse in evolutionary terms and contains chitinases enzyme from bacteria, fungi, viruses, animals, and some plant. Family 19 contains plant chitinases (classes I, II, and IV) and some *Streptomyces* chitinases. The chitinases of the both 18 and 19 families, do not share amino acid sequence similarity. They have completely different molecular mechanisms and 3-D structures and are therefore likely to have evolved from different ancestors. Family 20 consists of the β-N-acetylhexosaminidases from bacteria, *Streptomycetes* and humans (Dahiya *et al*., 2006).

2.3.2.1 Bacterial Chitinases

In bacterial chitinases, the chitin-binding domain can either be located in the amino terminal or in the carboxyl terminal domains of the enzyme. Most of the bacterial chitinases, which have been isolated and sequenced so far, are included in Family 18 of the glycosyl hydrolases. Bacterial chitinases consists of molecular weight ranging from 20 to 60 kDa are smaller than insect chitinases (40 to 85 kDa) which is similar to that of plant chitinases (40 to 85 kDa) (Garbe and Collin, 2012).

Bacterial chitinases are active over a wide range of pH and temperatures, depending on the source of the bacteria from which they have been isolated. For example, endochitinase from *Streptomyces violaceusniger* have an optimum temperature at 28°C and thermostable chitinase from *Streptomyces thermoviolaceus* OPC-520 have an optimum temperature at 80°C. Also, the enzyme has high pH optimum in the range of 8.0 to 10.5, while the chitinase isolated from *Stenotrophomonas maltophilia* C3 has a pH optimum in the range of 4.5 to 5.0. Bacterial chitinases also show a broad range of isoelectric points (pI 4.5 to 8.5).

It is considered that bacteria mainly produce chitinases in order to supply carbon and nitrogen as a source of nutrients. The enzyme production of chitinases in bacteria is mainly for the degradation of chitin and its

utilization as an energy source. The presence of multiple chitinase producing enzymes has been described in various microorganisms such as *Aeromonas species* 10S-24, *Pseudomonas aeruginosa* K-187, *Bacillus circulans* WL-12 (Aachmann *et al.*, 2012).

2.3.2.2 Fungal Chitinases

Fungal chitinases have multiple functions as they play a diverse role in nutrition, morphogenesis and fungal development processes. Chitin is a major cell wall component of fungi. Fungal chitinases show a high amino acid homology with class III plant chitinases. Mostly, they belong to the Family 18 of the glycosyl hydrolase superfamily. The basic structure of Family 18 fungal chitinases consists of 5 domains or otherwise known as 5 regions: (1) catalytic domain, (2) N-terminal signal peptide region, (3) chitin-binding domain, (4) serine/threonine rich-region and (5) C-terminal extension region (Taechowisan *et al*., 2003).

Other important applications of fungal chitinases include the possibility for improving plant resistance with the help of genetic manipulation techniques. The chi42 gene of *Trichoderma harzianum* encodes a powerful endochitinase, which has a much stronger anti-fungal activity against a number of phytopathogenic fungi and is expressed constitutively in potato, apple and tobacco. These transgenic plants thereby show a high level of resistance against phytopathogenic fungi. Fungal chitinases are also employed in insect control (Hamid *et al*., 2013).

2.3.2.3 Plant Chitinases

Chitinases are present in plant parts like stems, seeds, flowers and tubers. They are developmentally regulated as well as tissue-specific. Plant chitinases are produced as pathogenesis-related proteins in plant self defense in response to the attack of phytopathogens, or by contact with elicitors such as chito-oligosaccharides or growth regulators such as ethylene. There are some chitinases, which are expressed in response to environmental stresses like high salt concentration, cold and drought. There are also reports of some chitinases, which take part in vital physiological processes of plants, like

ethylene synthesis and embryogenesis. Chitinase is a polypeptide and a major pathogenesis-related protein, accumulates in the infected plant tissue extracellularly. The chitinases of plants can be detected during their development in the early stages of growth. The chitinases of plants are generally endochitinases of smaller molecular weight than the insect chitinases (Larsen *et al.*, 2011).

2.3.2.4 Insect Chitinases

The chitinases found in the insects have been described from *Bombyx mori* and *Manduca sexta*. These enzymes play very important role as degradative enzymes during ecdysis where endochitinases randomly break the chitin to chitooligosaccharides, then it is hydrolyzed by exoenzymes to N-acetyl-glucosamine. The monomer is reused for new chitin synthesis. Insect chitinases also play defensive roles against their own parasites and the enzyme production is regulated by hormones during the transformation of the larvae. Allosaminidin is the inhibitor of insect chitinases. Chitinases are also found in crustaceans like shrimps and prawns (Hamid *et al*., 2013).

2.3.2.5 Mammalian Chitinases

The immune response that may correlate with chitin recognition is the production of chitin-degrading enzymes, known as chitinases, by humans and other mammals. Chitinases belong to the glycosyl hydrolase Family 18, which is comprised of various proteins found in a wide range of organisms, including plants, bacteria, fungi, insects, protozoa and mammals (Vega and Kalkum, 2012).

2.3.3 Functions of Chitinase

Production of chitinase from the organism has several functions that can be used by organism itself and also by industrial sectors. In bacteria, chitinase play an important role in nutrition and parasitism while involving morphogenesis in fungi, protozoa and invertebrates. In addition, chitinases are also involved in the process of defence mechanism of plant and vertebrates. In bacteria, chitinase are considered primarily to digest and

hydrolyse chitin into carbon and nitrogen, which serves as a nutrient. On the other hand, chitinase act like catalyst in chitin degradation. Fungal chitinases are important for surviving of organism and it is synthesized itself. This is because they are involved in important morphogenic processes such as spore germination, hyphal elongation and hyphal branching (Das *et al*., 2012b).

2.4 Methods of Production of Chitinase

A huge number of methods have been used for the microbial chitinases production, which include fed-batch fermentation, continuous fermentation and liquid batch fermentation. Chitinase production is also diminished by some physical elements such as pH, aeration and incubation temperature. Chitinase production was stimulated in *Bacillus sp.* BG-11 subsequent to addition of amino acids and their analogs, for example tryptophan, tyrosine, glutamine, and arginine in the growth medium at a concentration of 0.1 mM. In order to improve the production of chitinases from different organisms, several methods were carried out such as biphasic cell systems, cell immobilization, solid-state fermentations and so on (Hamid *et al*., 2013).

2.4.1 Chitinase Production

Chitinase from microbial source has been produced by liquid batch fermentation, continuous fermentation and fed-batch fermentation. In addition to these, solid-state fermentation and biphasic cell systems have also been used for the production of chitinase. Generally, chitinase produced from microorganisms is inducible in nature.

Extracellular chitinase production is reported to be influenced by media components such as carbon sources, nitrogen sources and agricultural residues such as rice bran, wheat bran, etc. In an immobilized system, whole cell immobilization of an organism to a solid support such as polyurethane foam was applied. Chitinase production was enhanced up to 4.8-fold over a period of 72 hours in submerged fermentation.

2.4.2 Chitinase Inhibitors

In addition to general enzyme inhibitors, such as organic compounds and oxidizing or reducing agents, a number of reports are available on the natural chitinase inhibitors. An antibiotic like Allosamidin, produced by *Streptomyces species* is a known specific inhibitor of chitinases production from insects, yeast, fungi and human serum. Allosamidin is a competitive inhibitor. It is similar to N - acetylglucosamine but lacks a pyranose ring oxygen and contains an oxazoline ring in which the methyl group is substituted by dimethylamine.

Psammaplin A, a brominated tryrosine-derived compound, was found to be a noncompetitive inhibitor of chitinase production from *Serratia marcescens*, a Family 18 chitinase. Crystallographic studies suggest that a disordered Psammaplin A molecule is bound near the active site.

Another chitinase inhibitor, Argadin, was isolated from *Clonostachys species*, FO-7314. A new chitinase inhibitor, CI-4, was isolated from *Pseudomonas species* IZ08 (Dahiya *et al*., 2006).

2.5 Chitinase Induction and Regulation

Little is known about how host chitinase activity is induced, but there is some indication that chitinase production and chitin recognition could be linked, it is understood by the concept of MALDI-TOF and mass spectrometry to analyze the degradation of chitin by chitotriosidase and followed the stimulation of human monocyte or macrophage with a chitin hexamer. Portions of the downstream signaling pathway leading to chitinase expression have been analyzed. The upstream molecular signaling pathway that leads to *CHIT-1* gene activation and chitinase induction has not been determined; however, roles for some key proteins involved in chitinase regulation have been noted (Vega and Kalkum, 2012).

2.6 Applications of Chitinase

Chitinases may be used to convert chitin-containing biomass into useful (depolymerized) components (Lee *et al.*, 2011). Chitinases have shown

an immense potential for increasing the production of several useful products in the most economic way (Dahiya *et al*., 2006). The major applications of chitinases are discussed in the following subheadings:

2.6.1 Chitinase in Biocontrol of Plant Pathogenic Fungi and Insects

In many plant species, local invasion of the pathogen induces production of chitinases. As pathogenic fungi and insects contain chitin in their protective surface, induction of chitinases in plants is the main defense response. Most of these chitinases are induced in vegetative plant organs by infection but some of them also present in seeds. Number of soil bacteria also produces chitinolytic enzymes. Several species of fungi are very potent biocontrol agents of plant pathogenic fungi and also for insects. The mycoparasitic and entomopathogenic fungi produce chitinases for invasion and as one of the host killing components (Ghasemi *et al.*, 2011).

2.6.2 Chitinase as a Target for Biopesticides

Chitin is present in the exoskeleton and gut linings of insects. The insect chitinase has been described from *Bombyx mori*, *Manduca sexta* and several other species. Similarly, chitinases have been implicated in different morphogenetic events in fungi. The pseudotrisaccharide, allosamidin, is a potent chitinase inhibitor from most of the sources. The allosaminidin was found to be inhibitory after ingestion to the growth of mite, *Tetranychus urticae* and a larva of a housefly, *Myrothecium domestica*. However, there is no report for the inhibition of lepidopteran insects by oral or topical application. Nevertheless, chitinase inhibitors can be explored as potential biopesticides (Patil *et al*., 2000).

2.6.3 Mosquito Control

The worldwide socio-economical aspects of diseases spread out by mosquito make them potential targets for various pest control agents. In the case of mosquitoes, entomopthogenic fungus like *Beauveria bassiana* could not infect eggs of *Aedes aegypti,* a vector of yellow fever and dengue and other related species may be due to aquatic environment. The scarabaeid

eggs laid in the soil found to be susceptible to *B. bassiana*, *M. verrucaria,* a saprophytic fungus, produces a total complex of an insect cuticle degrading enzymes. It has been seen that both first and fourth instar of larvae of a mosquito, *A. aegypti*, can be killed within 48 hours with the help of the crude enzyme preparation from *M. verrucaria*. Though 100% mortality was observed with the crude enzyme (170-mg protein per liter) preparation within 48 hours, with purified endo-chitinase, lethal time (LT50) was 48 hours for first instar larvae and 120 hours for forth instar larvae. However, the time period was found to be decreased correspondingly to 24 hours and 48 hours when the purified chitinase was supplemented with lipolytic activity (Dahiya *et al*., 2006).

2.6.4 Production of Chito–oligosaccharides

There is a growing appreciation of the potential of biologically active chito-oligosaccharides. They act as elicitors of plant defense, involved in the signaling for the formation of root nodule and are potentially useful in human medicine too. For example, chitohexose and chitoheptose shows anti-tumor activity. A chitinase from *Vibrio alginolyticus* was used to prepare chitopentose and chitotriose from colloidal chitin. *N*,*N*9-Diacetylchitobiose has been widely used as a starting material for synthesis of biologically active compounds. Chitinase from *Bacillus* is used for the synthesis of chitobiose by combining N – acetylglucosamine and a sugar oxazoline derivative. The N–acetylglucosamine itself also is an anti-inflammatory drug (Patil *et al*., 2000).

2.6.5 Single Cell Protein (SCP) Production

For the effective utilization of chitinous waste, a parallel concept used for cellulose bioconversion to single cell protein (SCP) was suggested. Chitinase from *Sclerotium marcescens* to hydrolyze chitinous material and yeast, *Pichia kudriavzevii* for SCP that was acceptable for usage in aquaculture. In general, fungi used as the source for SCP are *Hansenula polymorpha, M. verrucaria, Candida tropicalis, S. cerevisiae* and so on. The criteria used to evaluate SCP production are growth yield, total protein and nucleic acid contents. The protein contents in the organisms used were

between 39 to 73% whereas the nucleic acid contents were 1–11%. The best reported was *S. cerevisiae* that exhibited 60% protein and 1 to 3% nucleic acid contents. The high *N*-acetylglucosaminidase activity in the culture filtrate of *M. verrucaria* yielded high levels of N - acetylglucosamine. The total protein contents were reported to be 61% with very low contents of nucleic acids (3.1%) (Sanders *et al.*, 2007).

2.6.6 Miscellaneous Application

Fungal protoplasts have gained substantial importance in the mycological research as strain improvement program for biotechnological applications. One of the major components of the fungal cell wall lysing enzyme complex is chitinase or chitosanase. It has been seen using various mycolytic enzyme preparations singly or in combination for protoplast isolation that, high chitinase levels permit effective fungal mycelia degradation. Enzymatic hydrolysis of the cell walls using chitinase preparation was found to be effective in the recovery of tannase enzyme. The enzyme like tannase is used in the food industry to remove the unwanted tannins and for producing gallic acid that is used as preservative (Patil *et al*., 2000).

2.6.7 Morphogenesis

Chitinases play an important role in yeast and insect morphogenesis. The role of chitinases in cell separation during growth in *S. cerevisiae*, functional expression of chitinase and chitosanase and their effects on morphogenesis in the yeast *S. pombe* has been studied. When the chiA gene was expressed in *S. pombe*, yeast cells grow slowly and cells become elongated, but when the choA gene was expressed, cells become swollen. Expression of both chiA and choA genes resulted in elongated and fat cells (Dahiya *et al*., 2006).

2.6.8 Medical Application

Chitooligosaccharides have an enormous pharmaceutical potential. They are involved in the signaling for root nodule formation, act as elicitors of plant defense and also have a potential to be used in human medicines (e.g.,

anti-tumor activity is shown by chitohexose and chitoheptose) (Hamid *et al*., 2013).

Chitinases can be employed in human health care, such as making ophthalmic preparations with chitinases and microbiocides. A direct medical use has been suggested for chitinases in the therapy for fungal diseases in potentiating the activity of antifungal drugs. They can also be used as potential additives in antifungal creams and lotions due to their topical applications (Dahiya *et al*., 2006).

A number of artificial medical articles such as contact lenses, artificial skin, and surgical stitches have been formed from chitin derivatives. These derivatives have an extensive medical use because quite a few of these chitin derivatives are known to be non-toxic, non-allergic, biocompatible and biodegradable. Chitinases also have some other medical applications as well. For example, first discovery of the involvement of acidic mammalian chitinase (AMCase) in the pathogenesis of asthma was novel and unexpected because of the fact that mammals do not use chitin as an energy source, nor do they produce any chitinous structure. Several lines of evidence have demonstrated the importance of chitinases as an effector of host defense in the mammalian immune system (Hamid *et al*., 2013).

2.6.9 Chitinase in Plant Defense

Plants represent the major component of biota and have the capability to synthesize their food through the process of photosynthesis. Physiological and environmental changes affect their health and make them vulnerable to variety of diseases thus directly or indirectly affect other components of ecosystem. A large number of environmental issues are linked with the eradication of plant diseases with chemical compounds. Most of these diseases are caused by fungal and insect pathogens. Chitin is the main structural component of these organisms and thus the enzyme responsible to hydrolyze chitin content are receiving attention in regard to their development as biopesticides or chemical defense proteins in transgenic plants and in microbial biocontrol agents. Therefore, understanding the overview of

chitinase will provide a basis for improving the pathogenic activity of potential biocontrol strains, for developing novel biological control strategies and for exploring their roles in the plant defense. The present review describes the properties of chitinase with respect to plant health improvement (Sharma *et al*., 2011).

2.7 Role of Chitinase

Different organisms produce a wide variety of hydrolytic enzymes that exhibit different substrate specificities and other properties useful for various functions. In bacteria, chitinases play roles in nutrition and parasitism whereas in fungi, protozoa and invertebrates they are also involved in morphogenesis. Chitinases are involved in the defense mechanism of plants and vertebrates. Baculoviruses, which are used for biological control of insect pests, also produce chitinases for pathogenesis. Chitinase activity in human serum has been recently described. The possible role suggested is a defense against fungal pathogens. An enzyme, chitotriosidase is used as a marker of Gaucher disease, a lysosomal storage disorder (Patil *et al*., 2000).

2.8 Future Prospects

In the future, there is a possibility of generating chitinases with novel functions. Chitinases can be exploited for their use as food preservatives, thereby increasing the shelf life of the foods. A vast understanding of the biological roles of different chitinases would help us to develop novel therapeutic approaches for several diseases including asthma and chronic rhinosinusitis. There is a possibility of using chitinases as anti-tumor drugs since chitohexose and chitoheptaose has shown an anti-tumor activity. These enzymes can be used for the enhancement of human the immune system. This research can be directed towards the identification of the active sites of chitinases and the novel functions associated with them. We can exploit protein engineering for the production of chitinases with exclusive functions (Hamid *et al*., 2013).

Experimental Procedure

3.0 EXPERIMENTAL PROCEDURE

Chitin, a polysaccharide of animal origin, is obtained from waste material of seafood industries. It is present in the skeletal material of crustaceans such as crabs, shrimps, prawns and crayfish. Chitin is degraded by the enzyme known as Chitinase. Chitinase hold great economic value due to their versatile biological activities and chemical applications, mainly in medical and pharmaceutical areas (Mathur *et al*., 2011). Chitinase are produced by a variety of microorganisms including many species of bacteria and fungi.

Chitin is hydrolyzed by the enzyme of chitinase, an unbranched polymer of β-1,4-N-acetylglucosamine. In recent years, soil-borne microorganisms that produce chitinases are considered as potential biocontrol agents against fungi and nematodes which cause diseases of agricultural crops. Chitinases also play an important physiological and ecological role in ecosystems as recyclers of chitin, by generating carbon and nitrogen sources. Many chitinases of varied organisms have been isolated and their corresponding genes were cloned (Kupiec and Chet, 1998).

The experimental procedure pertaining to the study **"Optimization of Chitinase Produced by Microorganisms Isolated from the Soil Degraded Crab Shell Waste"** was carried out under the following headings:

3.1 Collection of Microbial Source

3.2 Isolation, Maintenance and Identification of the Bacterial Strain

- **3.2.1 Isolation of the Bacterial Strain**
- **3.2.2 Screening of the Bacterial Strain**
- **3.2.3 Maintenance of the Cultures**
- **3.2.4 Identification of the Bacterial Strain**
  - **3.2.4.1 Gram Staining**
  - **3.2.4.2 Biochemical Tests**

3.3 Growth of the Bacterial Strain for Enzyme Production

3.4 Extraction and Estimation of the Activity of Enzyme

- **3.4.1 Chitinase**

3.5 Determination of Protein Content

3.6 Optimization of Chitinase Production

3.6.1 Incubation Period

3.6.2 Inoculum Concentration

3.6.3 Carbon Source

3.6.4 Nitrogen Source

3.6.5 pH

3.6.6 Metal Salts and Metal Ions

3.1 Collection of Microbial Source

For the present study, chitinase producing microorganisms were collected in the form of soil degraded crab shell and soil sample was collected from crab shell disposal site in Coimbatore. The samples were shown in the Plates 1 and 2.

The soil sample were collected near the places where the crab shells are discharged from the market. Both soil degraded crab shell waste and soil sample were stored in a sterile ziplock covers and brought to the laboratory. Both the samples were maintained at 4ºC and used for further procedures.

PLATE 1

Soil Sample from Waste from Disposal site

PLATE 2

Soil Degraded Crab Shell Disposal Site

3.2 Isolation, Maintenance and Identification of the Bacterial Strain

3.2.1 Isolation of the Bacterial Strain

Crab shell waste and soil samples were serially diluted 7 – folds (10^{-1} to 10^{-7}) and each of the dilution was spread plated on tryptone soy agar medium and incubated at 37ºC for 24 hours. The grown individual colonies were screened and poured into Nutrient agar medium and incubated for 24 hours at 37ºC. The composition of Nutrient agar medium is detailed in Appendix I. Morphologically different colonies were identified. The detailed procedure is given in Appendix II.

3.2.2 Screening of the Bacterial Strain

The morphologically different bacterial colonies were streaked on colloidal chitin agar medium. The plates were incubated at 37ºC for 96 hours. After the incubation the strains gave a larger hydrolysis zone which indicates the positive reaction. The detailed protocol is described in Appendix III. Further maximum enzyme production was then obtained in nutrient broth medium.

3.2.3 Maintenance of the Cultures

The isolated bacterial colonies were streaked on nutrient agar slants and subcultured periodically. The cultures were maintained at 4ºC.

3.2.4 Identification of the Bacterial Strain

The isolated colonies were studied for their morphological, physiological and biochemical characteristics.

3.2.4.1 Gram Staining

The bacterial colonies were identified by Gram staining technique. The procedure is given in Appendix IV.

3.2.4.2 Biochemical Tests

The isolated bacterial colonies were further characterized by performing the various biochemical tests. The procedures are described in the Appendix V.

3.3 Growth of the Bacterial Strain for Enzyme Production

For bacterial cultures, the test for the chitinase production was carried out in the flasks containing 100 ml nutrient broth. The composition of the nutrient broth is given in Appendix I. The flask containing broth were incubated at 37ºC in a shaker for 24 hours.

3.4 Extraction and Estimation of the Activity of Enzyme

The 24 hours old liquid culture was harvested (over night culture for bacteria) with bacterial species. The cells were separated by centrifuging at 1000 rpm for 20 minutes at 4ºC. The supernatant obtained was used as crude enzyme extract.

3.4.1 Chitinase

The activity of chitinase was determined by measuring the reducing sugar released from colloidal chitin as per the method of Aida and Taghreed (2014). The procedure is explained in Appendix VI.

3.5 Determination of Protein Content

The protein content in the crude enzyme extract was determined using Lowry's method. The detailed protocol is explained in the Appendix VII.

3.6 Optimization of Chitinase Production

3.6.1 Incubation Period

24 hours old culture was inoculated into four test tubes containing 15 ml of nutrient broth marked as 24 hours, 48 hours, 72 hours and 96 hours and were used to determine the optimum time or incubation period required for

maximum enzyme activity. Tubes were incubated at 37˚C on a rotary shaker at respective period. After each incubation period the cultures were used for biomass determination (Appendix VIII). The enzyme was extracted and the activity of chitinase (Appendix VI) and protein content (Appendix VII) were determined.

3.6.2 Inoculum Concentration

The inoculum size was standardized by evaluating the effect of varying inoculum size for seeding the basal medium. 24 hours old culture was used as the inoculum. 100ml of nutrient broth in 6 conical flasks were taken and inoculated with 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml and 5.0 ml of the inoculum. The conical flasks were incubated at 37ºC for 48 hours. The obtained culture was used for the determination of biomass **(**Appendix VIII). After the enzyme was extracted, the activity of chitinase (Appendix VI) and the protein content (Appendix VII) were determined.

3.6.3 Carbon Source

The effect of carbon source on the chitinase yield was analyzed by adding various carbon sources like dextrose, sucrose, glucose, lactose, maltose and starch in the basal medium (Appendix I). After 48 hours the biomass was determined (Appendix VIII). The enzyme was extracted and determined for their activity of chitinase (Appendix VI) and protein content (Appendix VII).

3.6.4 Nitrogen Source

The medium was supplemented with different nitrogen sources for analysis of the maximum chitinase production. Nutrient broth usually contains nitrogen source as peptone and beef extract. Instead of peptone in nutrient broth other nitrogen sources like tryptone, casein, ammonium oxalate and instead of beef extract, ammonium sulphate, ammonium nitrate and potassium nitrate were used (1% w/v). After 48 hours of incubation the biomass was determined (Appendix VIII). The enzyme was extracted to find the chitinase activity (Appendix VI) and protein content (Appendix VII).

3.6.5 pH

A wide pH range of 5 to 10 was used to determine the optimum pH for chitinase production. For the analysis, 100 ml of nutrient broth in 6 conical flasks were taken. The pH medium was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0 and 10 respectively using 0.1 N HCl and 0.1 N NaOH. 24 hours old culture was used as the inoculum. After 48 hours of incubation, biomass determination was carried out (Appendix VIII). From the enzyme extracted, the chitinase activity (Appendix VI) and protein content (Appendix VII) were determined.

3.6.6 Metal Salts and Metal Ions

The effect of various metal ions like ferric chloride, lead acetate, potassium chloride and calcium chloride were supplemented separately at a concentration of 10 mM in the nutrient broth. The concentrations of metal salts like sodium chloride of 5%, 10%, 15% are added into the nutrient broth. The inoculum used was 24 hours old culture. After 48 hours of incubation, the biomass was determined (Appendix VIII). The enzyme was extracted to analyze the chitinase activity (Appendix VI) and protein content (Appendix VII).

Results and Discussion

4.0 RESULTS AND DISCUSSION

Chitin is widely distributed in nature, particularly as a structural polysaccharide in fungal cell walls, the exoskeletons of arthropods, the outer shells of crustaceans and nematodes. Approximately 75% of the total weight of shellfish such as shrimp, crab, and krill, is considered waste.

Chitin comprises 20–58 % of the dry weight of this waste. Chitin has a broad range of applications in the biochemical, food and chemical industries and shows antimicrobial, anticholesterol and antitumor activity. Chitin and its related materials are also used in wastewater treatment, drug delivery, wound healing and also as dietary fiber. Waste from the processing of marine crustaceans is a significant commercial source of chitin (Brzezinska *et al*., 2013).

Microbial chitinase has been produced by liquid fermentation processes (batch, continuous and fed-batch fermentation) and is commercially available at a high cost. Generally, the production is controlled by physical factors (aeration, pH and incubation temperature) and by the growth media components (Rebah and Miled, 2013).

Chitinase find wide application for various industrial and biotechnological processes. In order to facilitate the use of enzyme for different industrial applications there is a need for chitinase with suitable biochemical properties. Hence the present study was an attempt to isolate chitinase producing microbes from soil sample and soil degraded crab shell waste. The samples collected from the crab shell disposable site served as the source for the isolation of chitinase producing microbes. The microbes were isolated and screened for their chitinase producing properties, followed by the optimization of conditions for maximum chitinase production.

The result obtained for the present study entitled **"Optimization of Chitinase Produced by Microorganisms Isolated from the Soil Degraded Crab Shell Waste"** are discussed under the following headings:

4.1 Isolation of Microorganisms

From the sample, the colonies were isolated on the tryptone soy agar plates. The bacterial strains isolated were shown in Table 1 and 2. Single morphologically distinct colony were obtained from the serially diluted soil sample namely CSS 10^{-2} whereas six morphologically distinct colonies were obtained from the serially diluted soil degraded crab shell waste samples namely CSW 10^{-1}, CSW 10-^{2}, CSW 10^{-3} (2 colonies), CSW 10^{-4}, CSW 10^{-5}. The morphologically distinct colonies for the selected samples were shown in Plate 3 and 4.

These morphologically distinct colonies were subcultured on nutrient agar plates and nutrient agar slants to get single isolate and stored at 4^{0}C (Plate 5 and 6).

Table 1

Isolation of the Bacterial Strain from Soil Samples

S.No	Serial Diluted Samples	Selected Strain	Name of the Strain
1.	CSS 10^{-1}	-	-
2.	CSS 10^{-2}	1	CSS21
3.	CSS 10^{-3}	-	-
4.	CSS 10^{-4}	-	-
5.	CSS 10^{-5}	-	-
6.	CSS 10^{-6}	-	-
7.	CSS 10^{-7}	-	-

***CSS – Crab Shell Soil**

From the above Table 1, the soil samples showed the similar morphological colonies on the tryptone soy agar plates. But in the sample CSS 10^{-2} show the best morphological colony known as CSS21.

Plate 3

Morphologically Distinct Colonies showed in Typtone Soy Agar Plate from Soil Samples

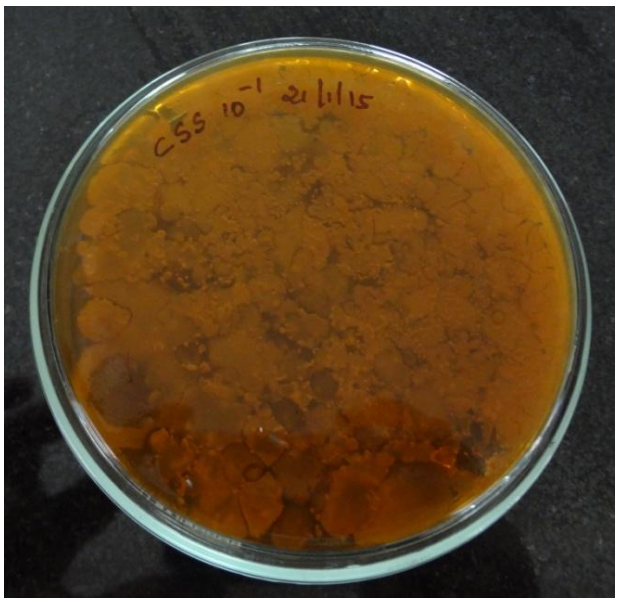


Table 2

Isolation of the Bacterial Strain from Crab Shell Waste Samples

S.No	Serial Diluted Samples	Selected Strain	Name of the Strain
1.	CSW 10^{-1}	1	CSW11
2.	CSW 10^{-2}	1	CSW21
3.	CSW 10^{-3}	2	CSW31 and CSW32
4.	CSW 10^{-4}	1	CSW41
5.	CSW 10^{-5}	1	CSW51
6.	CSW 10^{-6}	-	-
7.	CSW 10^{-7}	-	-

***CSW – Crab Shell Waste**

From Table 2, it is noted that CSW 10^{-1}, CSW 10^{-2}, CSW 10^{-4} and CSW 10^{-5} showed single morphological colonies separately and named as CSW11, CSW21, CSW41 and CSW51. Whereas CSW 10^{-3} produce the two different type of colonies based on their morphological characterization named as CSW31 and CSW32. But the CSW 10^{-6} and CSW 10^{-7} do not show any colony on the plates.

Plate 4

Morphologically Distinct Colonies showed in Typtone Soy Agar Plate from Soil Degraded Crab Shell Waste Samples

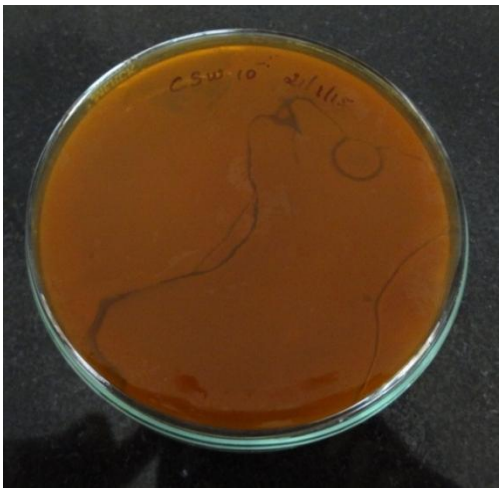


Plate 5

Pure Cultures of Single Bacterial Isolates showed in Nutrient Agar Plates from Soil Samples

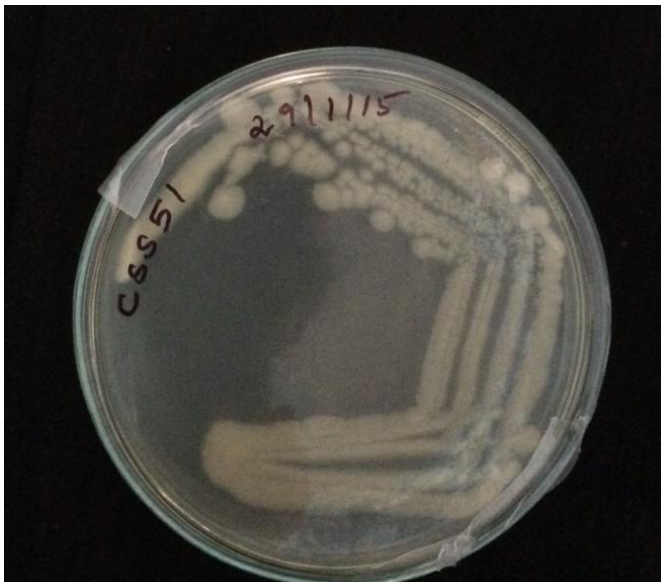
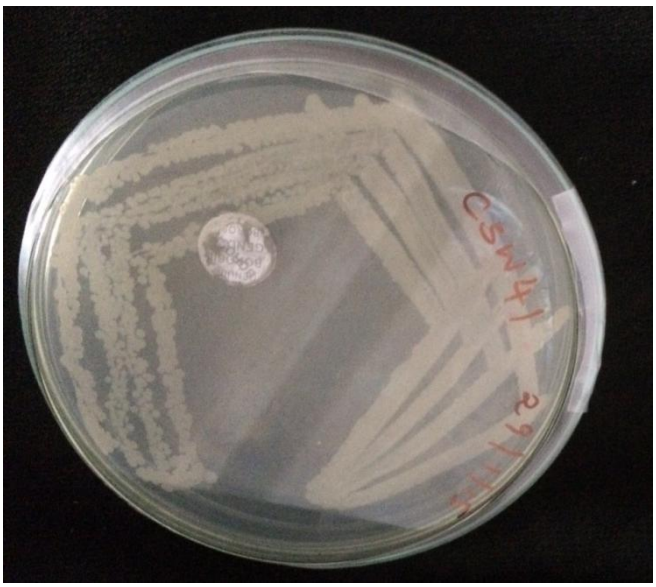


Plate 6

Pure Cultures of Single Bacterial Isolates showed in Nutrient Agar Plates from Soil Degraded Crab Shell Waste Samples



4.2 Screening for Chitinase Producing Microorganisms

The colonies were selected by serial dilution and it was further proceeded for chitinase screening using colloidal chitin agar medium. The positive cultures were tabulated in Table 3 and the positive results indicate the zone of clearance around the colony which is presented in Plate 7.

Table 3

Chitinase Producing Bacterial Strain from the Isolated samples

S.No	Bacterial Strain	Colloidal Chitin Agar Medium
1.	CSS21	Positive
2.	CSW11	Positive
3.	CSW21	Positive
4.	CSW31	Positive
5.	CSW32	Positive
6.	CSW41	Positive
7.	CSW51	Positive

From Table 3, out of 7 morphologically different bacterial strains that have been isolated from the soil and soil degraded samples, all the isolated samples were showing positive result in colloidal chitin agar medium.

Screening for chitinase producing microorganisms was supported by Jholapora *et al*. (2013a), the colonies which were detected in viable count studies were screened for chitinase was performed by spot inoculating each of the isolates at the center of Colloidal Chitin Agar (CCA) media plates. The colony was observed by zone of clearance around the colony.

Plate 7

Screening for Chitinase Producing Bacteria

Plate 7 indicates the bacterial growth on colloidal chitin agar medium and the positive reaction represented the clear zone surrounded by the bacterial colony.

4.3 Identification of Isolated Microorganisms

The isolated colonies were studied for their morphological and biochemical characteristics.

4.3.1 Gram Staining

The isolated cultures were identified using Gram staining method. The Gram staining test was summarized in Table 4.

Table 4

Gram Staining

S.No	Samples	Gram Staining	Shape	Colour	Organisms
1.	CSS21	+	Rod	Purple	*Bacillus*
2.	CSW11	-	Cocci	Pink	*Neisseria Meningitidis*
3.	CSW21	+	Rod	Purple	*Bacillus*
4.	CSW31	-	Cocci	Pink	*Neisseria meningitidis*
5.	CSW32	-	Cocci	Pink	*Neisseria meningitidis*
6.	CSW41	-	Rod	Pink	*Escherichia coli*
7.	CSW51	-	Rod	Pink	*Escherichia coli*

Table 4 represents the result of Gram staining. From the seven strains, two strains namely CSS21 and CSW21 were identified to be *Bacillus*, three strains CSW11, CSW31 and CSW32 were found to be as *Neisseria meningitidis* and remaining two strains CSW41 and CSW51 were identified as *Escherichia coli.*

4.3.2 Biochemical Tests

The isolated cultures were analyzed and identified based on the biochemical tests. The results obtained from the biochemical tests namely indole test, methyl red test, voges proskauer test, citrate utilization test, oxidase test, catalase test, starch hydrolysis test, carbohydrate fermentation test, triple sugar iron test, nitrate reduction test and urea hydrolysis test were summarized in Table 5 and Plate 8 - 17.

Table 5

Biochemical Tests

S.No	Tests	Samples						
		CSS 21	CSW 11	CSW 21	CSW 31	CSW 32	CSW 41	CSW 51
1.	Indole Test	-	-	-	+	+	-	-
2.	Methyl Red Test	-	+	-	+	+	+	+
3.	Voges Proskauer Test	-	+	+	-	-	-	-
4.	Citrate Utilization Test	+	+	+	-	+	-	-
5.	Oxidase Test	+	-	-	+	-	+	-
6.	Catalase Test	+	+	+	+	+	-	-
7.	Starch Hydrolysis Test	+	+	+	-	-	-	+
8.	Carbohydrate Fermentation Test	+	+	+	-	+	+	-
9.	Triple Sugar Iron Test	+	+	+	-	+	-	+
10.	Nitrate reduction Test	+	+	-	-	+	+	-
11.	Urea Hydrolysis Test	-	+	+	-	+	+	+

From Table 5, it is seen that microorganisms were identified based on the eleven biochemical tests. In that, CSW31 and CSW32 were shows the positive results of indole test. In methyl red test CSW11, CSW31, CSW32, CSW41 and CSW51 showed the positive results. Whereas in voges proskauer test two cultures namely CSW11 and CSW21 produced positive results. CSS21, CSW11, CSW21 and CSW32 were showing the positive results in citrate utilization test.

From the oxidase test of all isolated samples, only three cultures (CSS21, CSW31 and CSW41) were showing the positive reaction. From all the isolated cultures, CSW41 and CSW51 did not show any positive reaction for catalase test whereas remaining cultures show positive result. CSS21, CSW11, CSW21 and CSW51 showed positive result for starch hydrolysis test.

For carbohydrate fermentation test, CSS21, CSW11, CSW21, CSW32 and CSW41 showed positive results. Triple sugar iron test is another biochemical characterization test, which showed positive result for the isolated samples CSS21, CSW11, CSW21, CSW33 and CSW51 were seen. CSS21, CSW11, CSW32 and CSW41 shows positive result for nitrate reduction test. In urea hydrolysis test, the positive results of following cultures as CSW11, CSW21, CSw32, CSW41 and CSW51 were seen.

Biochemical Tests

Plate 8

Indole Test

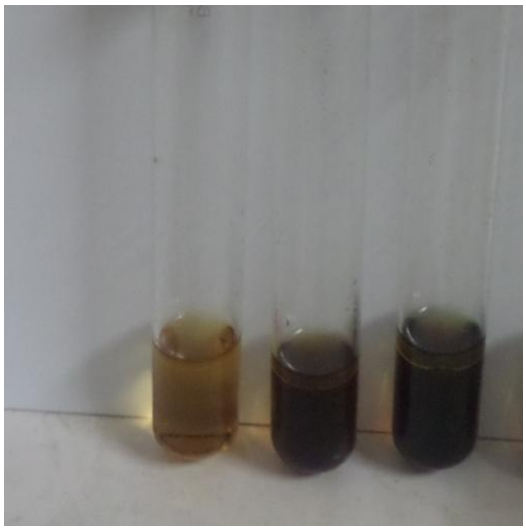


Plate 9

Methyl Red Test

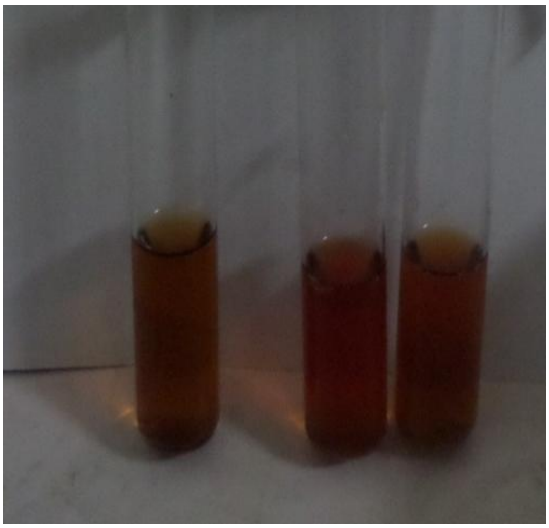


Plate 10

Voges Proskauer Test

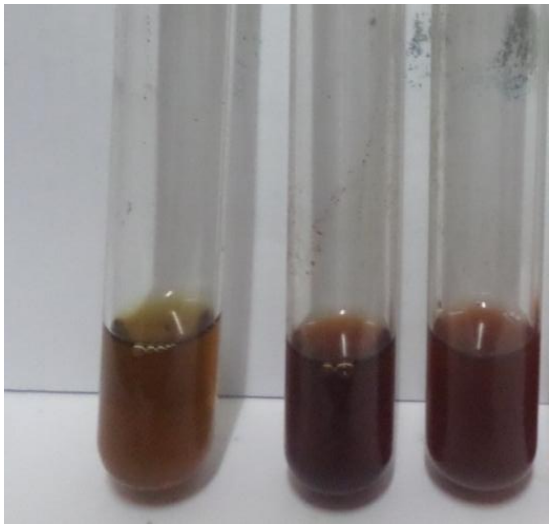


Plate 11

Citrate Utilization Test

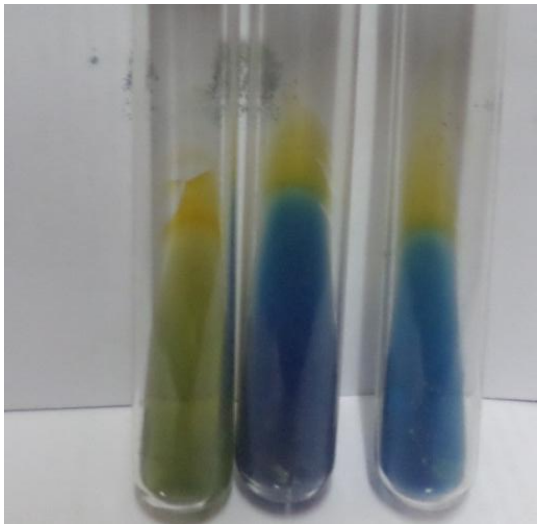


Plate 12

Oxidase Test

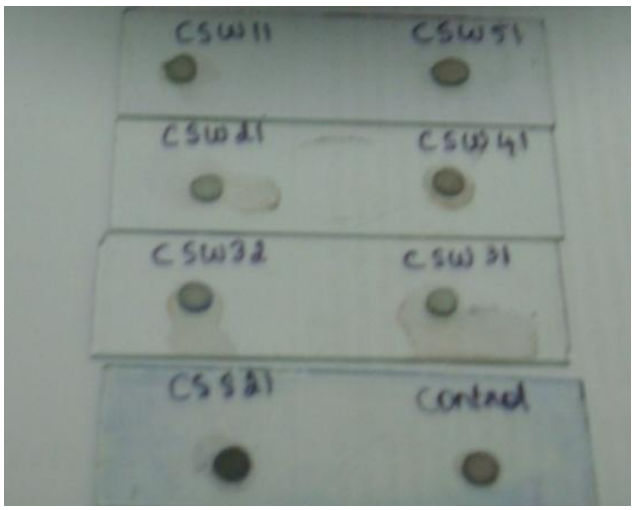


Plate 13

Starch Hydrolysis Test

Plate 14

Carbohydrate Fermentation Test

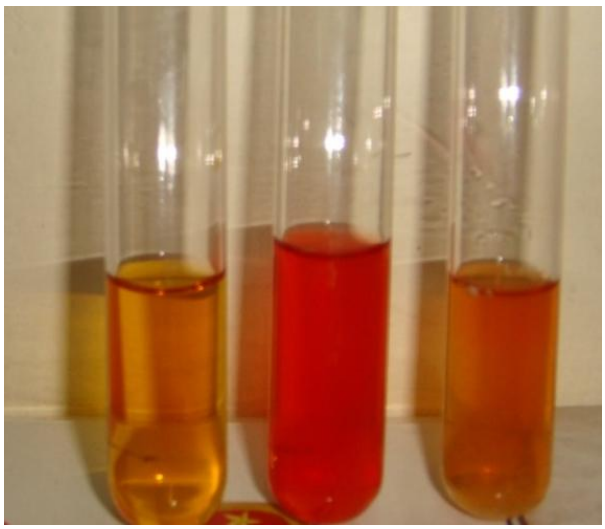


Plate 15

Triple Sugar Iron Test

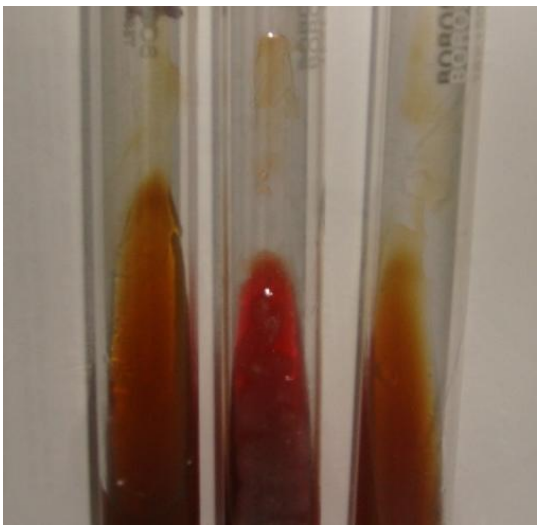


Plate 16

Nitrate Reduction Test

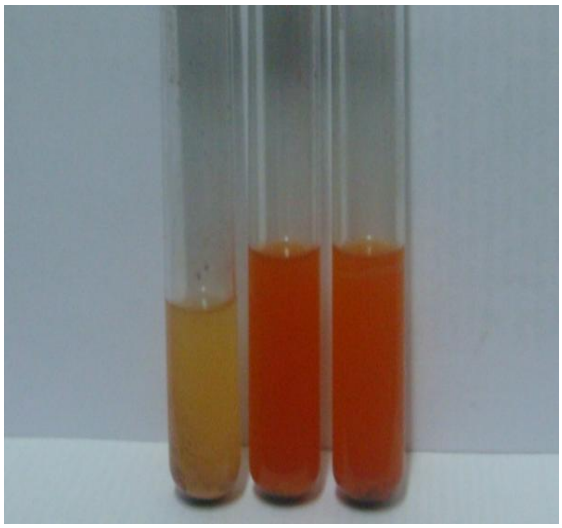


Plate 17

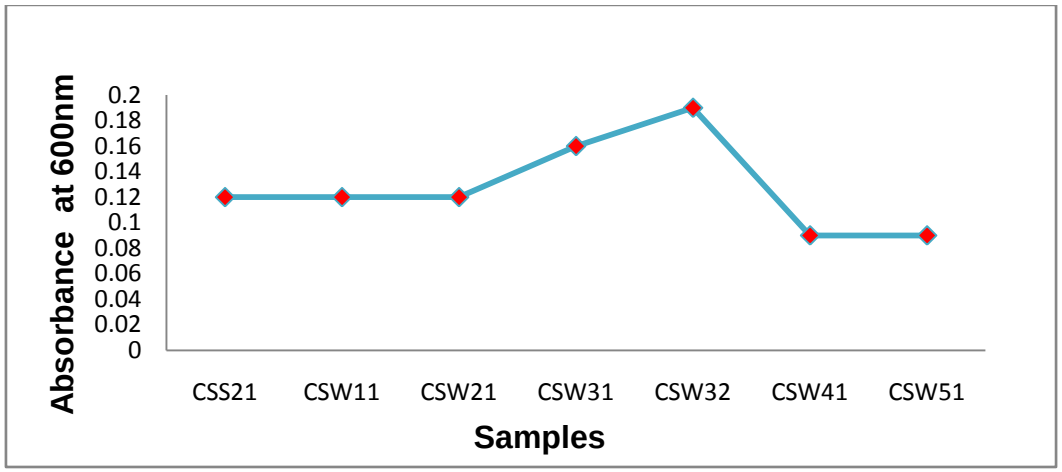
Urea Hydrolysis Test

4.4 Biomass Accumulation and Assay for Chitinase

Figure 1 shows the biomass accumulation of crude samples of different strains.

Figure 1

Biomass Accumulation on Crude Samples

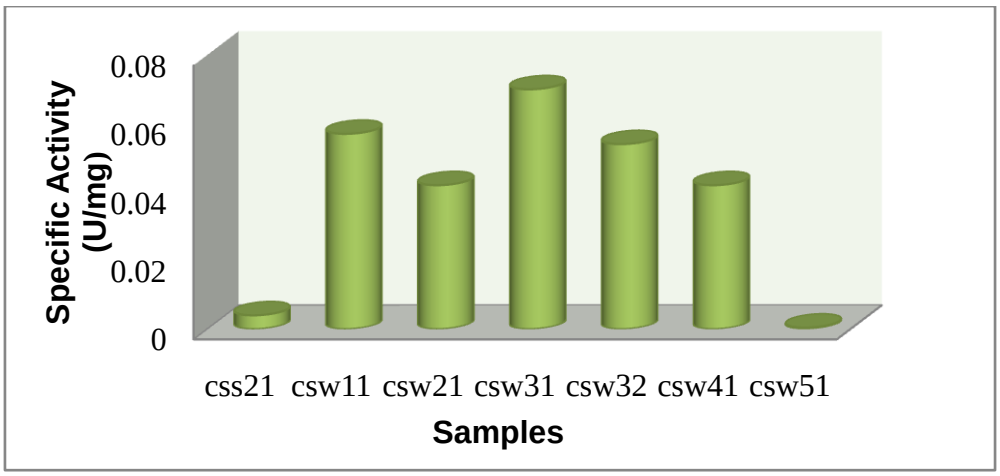


From Figure 1, it is clear that the highest biomass accumulation was obtained for CSW 32 culture of the crude sample which was followed by CSW31.

Figure 2 records the specific activity of crude samples.

Figure 2

Specific Activity of Crude Samples



From Figure 2, it can be interpreted that maximum chitinase production was observed for CSW 31 crude sample.

Brzeniska *et al*. (2013) had reported that the soil bacteria exhibited the highest activity in a culture medium containing colloidal chitin. Mahata *et al*. (2008) has reported that highest chitinase activity from isolate 99 was found on the fourth day of incubation while that from *Enterobacter* species G-1 on the third day. Chitinase activity from isolate 99 was 0.039 U/ml and its specific activity was 0.56 U/mg protein. Chitinase activity from isolate 99 and its specific activity were higher than those of *Enterobacter* species G-1 which were 0.029 U/ml and 0.48 U/mg protein.

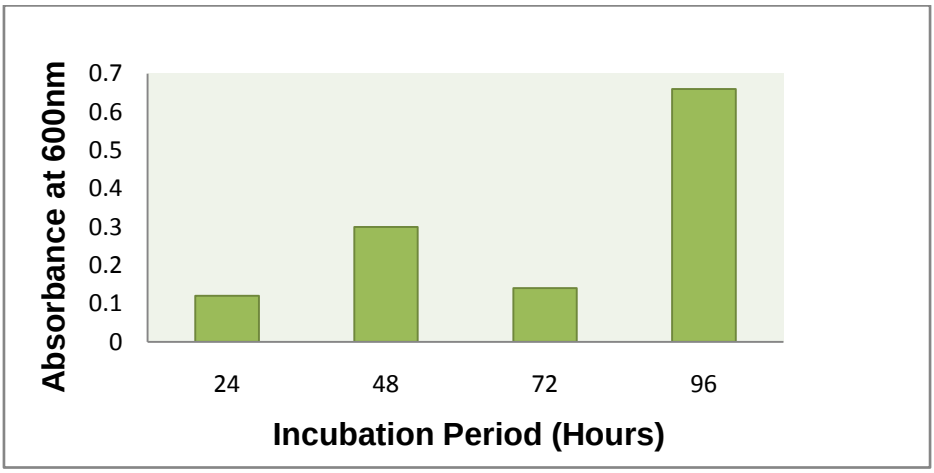
4.5 Optimization of Chitinase Production

4.5.1 Incubation period

The effect of incubation period on the biomass accumulation and enzyme production is depicted in Figures 3 and 4 respectively.

Figure 3

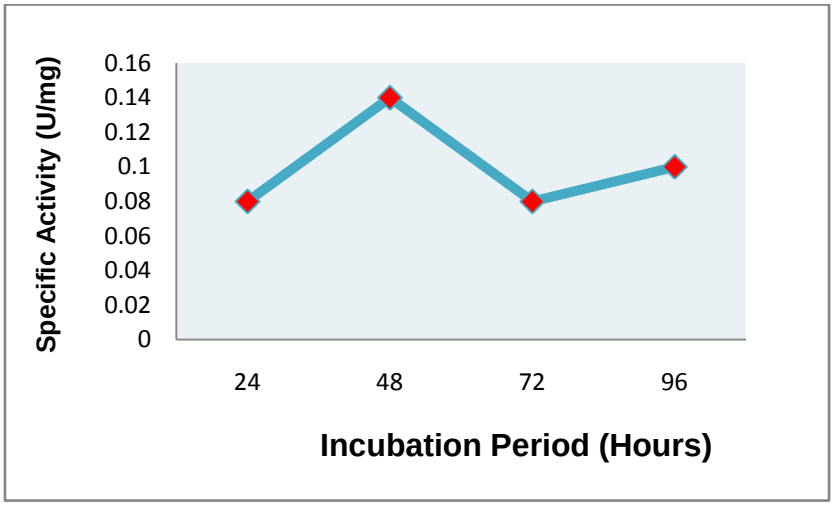
Effect of Incubation Period on Biomass Accumulation



From Figure 3, it is clear that highest biomass accumulation was obtained at 96 hours of incubation.

Figure 4

Effect of Incubation Period on the Activity of Chitinase



From Figure 4, it is noted that maximum chitinase production was observed at 48 hours.

This was in accordance to the study of Kuddus and Ahmad (2013) who reported that 48 hours culture of *Aeromonas hydrophila* and *Aeromonas punctata,* exhibited maximum chitinase activity. Other studies by Sujatha and Ammani (2013), Shanmugaiah *et al*. (2008) and Mahata *et al*. (2008) showed that chitinase production was gradually increased and peaked at 96 hours and was found to be decline gradually after 96 hours.

Das *et al*. (2012a) had suggested that best *Bacillus amyloliquefaciens* SM3 were nutrient broth with 0.5% colloidal chitin, pH 7.5, temperature 35°C with 72 h of incubation produce a maximum chitinase production.

4.5.2 Inoculum Concentration

The effect of inoculum concentration on biomass accumulation and chitinase production were depicted in Figures 5 and 6 respectively.

Figure 5

Effect of Inoculum Concentration on Biomass Accumulation

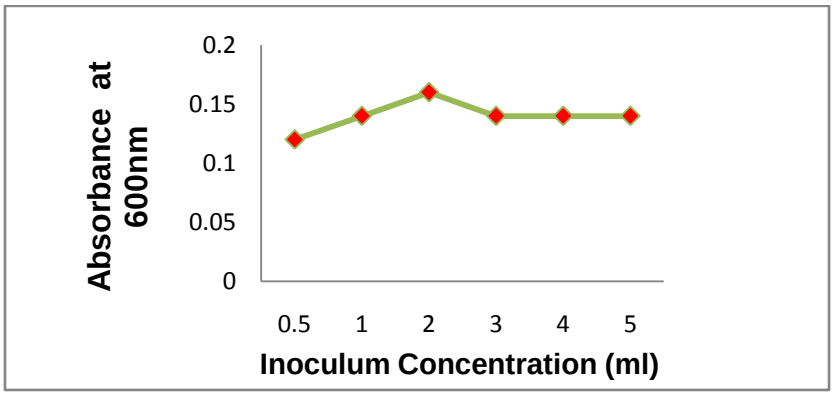
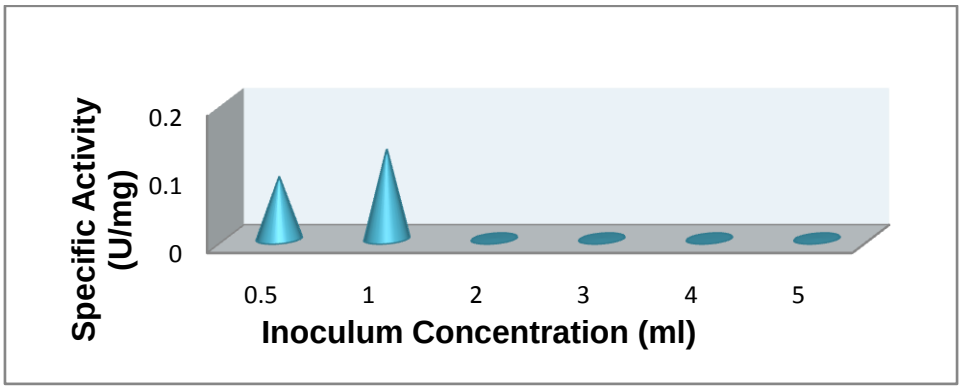


Figure 5 shows the effect of inoculum concentration on biomass accumulation. Higher growth of microbial culture was observed when 2 ml of inoculum concentration was used when compared with other inoculum concentrations.

Figure 6

Effect of Inoculum Concentration on the Activity of Chitinase



From Figure 6, it can be interpreted that maximum chitinase production was observed in 1 ml of inoculum followed by 0.5 ml. It denotes that when the inoculums concentration was increased above 1 ml the chitinase production started to decrease.

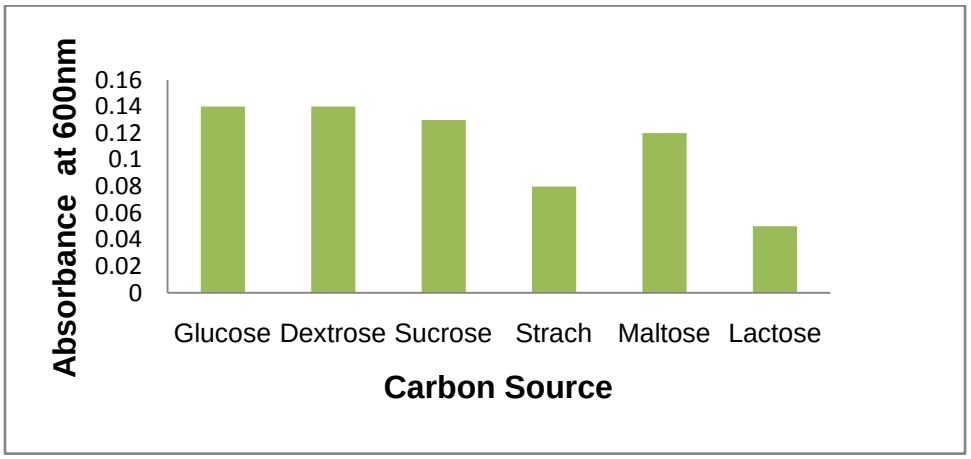
The results are in agreement with the results of Aida and Taghreed (2014) who had reported that 2% inoculum concentration was appropriate for chitinase production. This was also supported by the study of Bansode and Bajekal (2006) who had shown that chitinase production reached its maximum value with 0.5 ml of inoculums after seven days.

4.5.3 Carbon source

To find the optimum carbon source for maximum biomass accumulation and chitinase production, different carbon sources were supplemented in the medium and the results were interpreted in Figures 7 and 8 respectively.

Figure 7

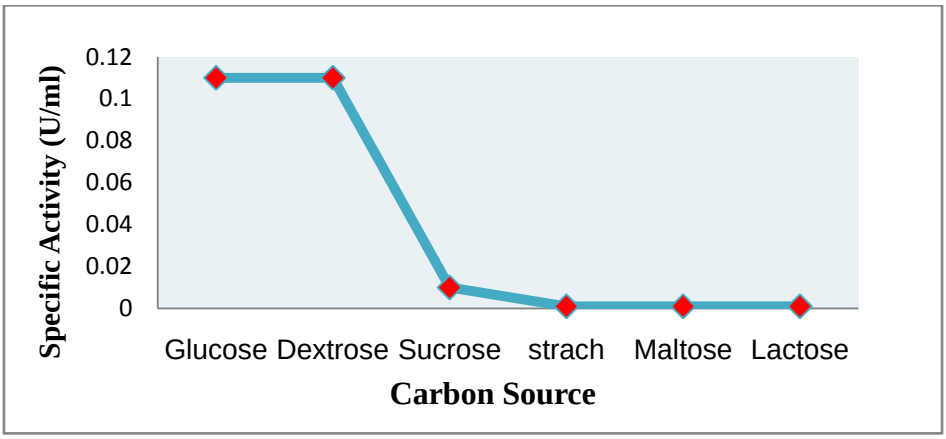
Effect of Carbon Source on Biomass Accumulation



From Figure 7, it is understood that maximum biomass accumulation occurred when glucose and dextrose was used, followed by sucrose and maltose.

Figure 8

Effect of Carbon Source on the Activity of Chitinase



From Figure 8, it is understood that maximum chitinase activity was obtained when the medium was supplemented with glucose and dextrose. The activity was seen to be lowered when sucrose is supplemented and the activity is even more decreased when it is supplemented with starch, maltose and lactose.

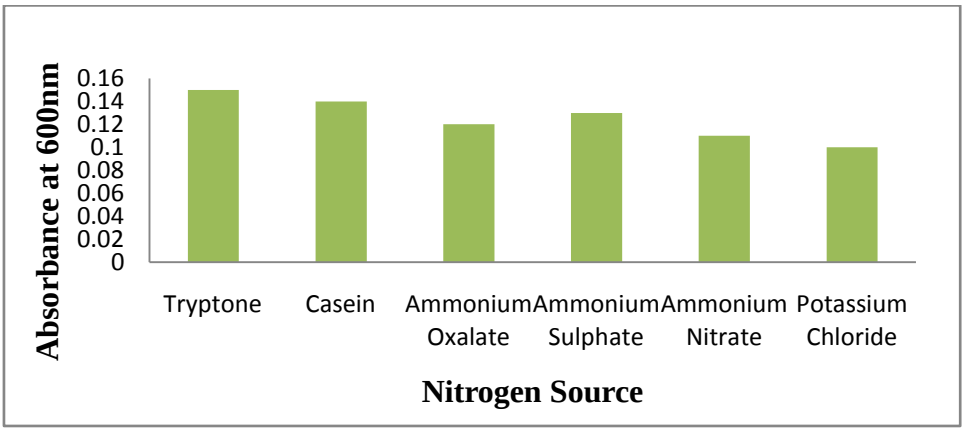
The study of Abdel-Aziz *et al*. (2012), Hirano *et al*. (2011) and Taechowisan *et al*. (2003) suggested that good carbon source for chitinase production was observed when the medium was supplemented with N-acetylglucosamine, where the production of chitinase was maximum, when compared with other carbon sources.

4.5.4 Nitrogen source

To optimize the nitrogen source for maximum biomass accumulation and chitinase production, different nitrogen sources were supplemented in the medium and results were interpreted in Figures 9 and 10 respectively.

Figure 9

Effect of Nitrogen Source on Biomass Accumulation



From the above Figure 9, it is clearly understood that the biomass accumulation was highest when tryptone was used as the nitrogen source. Considerable amount of biomass was also obtained in the medium supplemented with casein followed by ammonium sulphate.

Figure 10

Effect of Nitrogen Source on the Activity of Chitinase

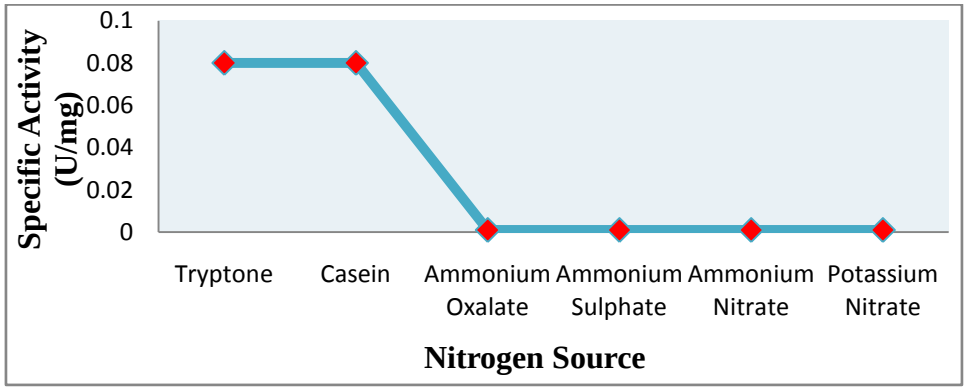


Figure 10 clearly indicate that the chitinase activity was highest when tryptone and casein was used as the nitrogen source. The activity was seen to be even more decreased when supplemented with ammonium oxalate, ammonium sulphate, ammonium nitrate and potassium nitrate.

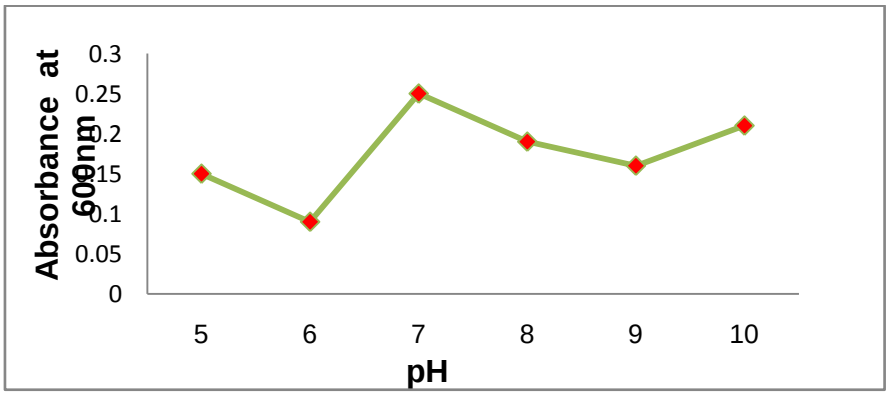
This was in accordance to the study of Khan and Khan (2014) and Jholapara *et al*. (2013b) who has shown that peptone and tryptone are good nitrogen supplements for chitinase production than ammonium sulphate and ammonium chloride.

4.5.5 pH

The effect of pH on biomass accumulation and chitinase production is depicted in Figures 11 and 12 respectively.

Figure 11

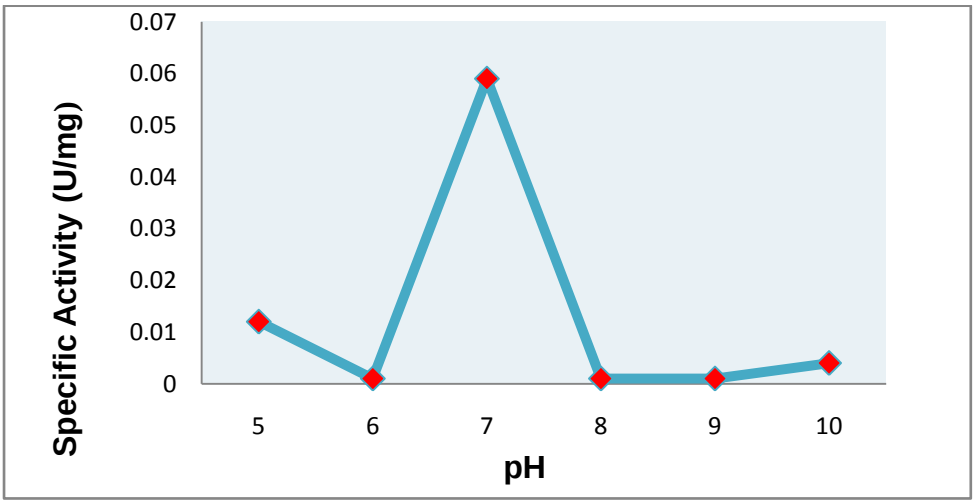
Effect of pH on Biomass Accumulation



It is evident from the above Figure 11, that the best pH for biomass accumulation was pH 7.0.

Figure 12

Effect of pH on the Activity of Chitinase



From the above Figure, the highest chitinase activity was found to be at pH 7 when compared to other pH.

This was in accordance to the study of Jholapara *et al*. (2013b) who shows the influence of initial pH on the culture medium. Maximum chitinase production was observed over a range of pH 6.0-7.0. At pH higher and lower than the optimum pH range, enzyme production was repressed.

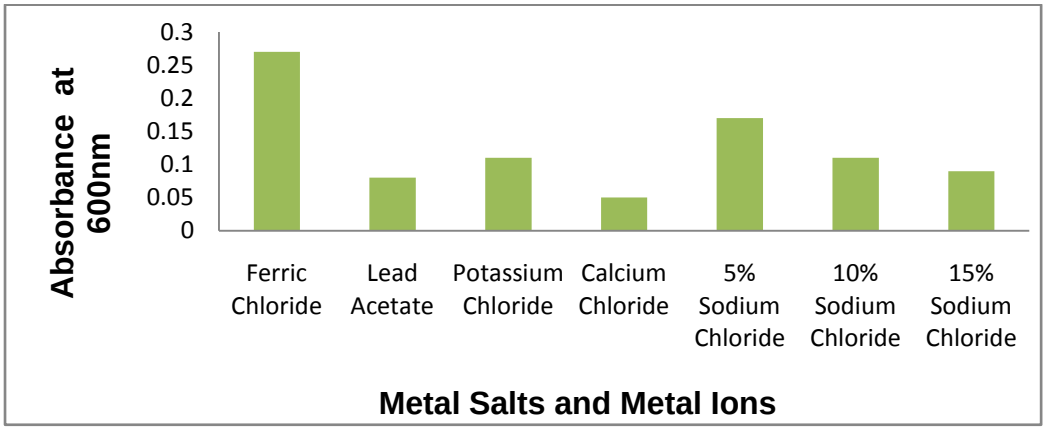
This was also supported by the study of Wang and Hwang (2001) who showed that the maximum amount of chitinase was produced by *Bacillus cereus*, *B. alvei*, and *B. sphaericus*, was observed at pH 7.0, pH 9.0 and pH 9.0 respectively. Whereas chitinase produced from *Pseudomonas aeruginosa* had pH 7.0 for the maximum enzyme production.

4.5.6 Metal Salts and Metal ions

Effect of metal salts and metal ions on biomass accumulation and enzyme production was interpreted in Figures 13 and 14 respectively.

Figure 13

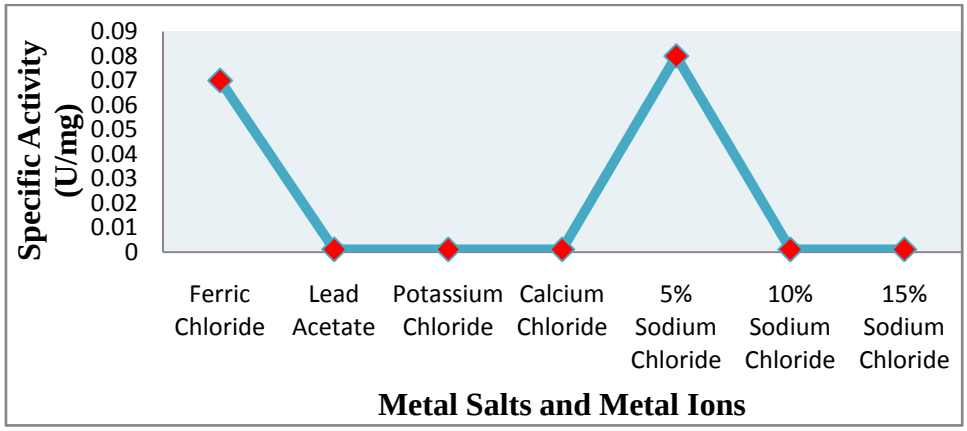
Effect of Metal Salts and Metal Ions on Biomass Accumulation



It can be clearly understood from Figure 13, in the various concentrations of sodium chloride used, maximum biomass accumulation was observed in production of media containing 5% sodium chloride. As the concentration of sodium chloride is increased, the biomass rate is declined. Among the metals used ferric chloride produced maximum biomass accumulation.

Figure 14

Effect of Metal Salts and Metal Ions on the Activity of Chitinase



From the above Figure 14, it is clearly understood that the enzyme activity were found to be highest in the medium containing 5% sodium chloride when compared to 10% and 15% of sodium chloride. Chitinase activity was also higher in the medium supplemented with ferric chloride when compared to other metal ions and salts.

Jholapara *et al*. (2013b) reported that the maximum enzyme activity is induced by 0.6% magnesium sulphate in a basal medium. Similar study of Thiagarajan *et al*. (2011) who has shown that the study reported that aluminium chloride and zinc sulphate enhanced the enzyme activity whereas mercuric chloride and mercuric nitrate inhibited the chitinase activity.

Mahata *et al*. (2008) who reported that the chitinase obtained from *Enterobacter* species was activated by copper sulphate but inhibited by mercuric chloride.

From the study it is clearly noted that the soil degraded crab shell waste sample had a better enzyme activity and protein content than the soil sample. Biochemical tests and Gram staining revealed the presence of three different types of microorganisms in the soil and soil degraded crab shell waste samples. By determination of specific activity of the chitinase enzyme, the CSW31 culture which is *Neisseria meningitidis* had a significant activity.

When different conditions was used for the optimization of CSW31 culture of *Neisseria meningitidis,* the best enzyme activity was recorded, which is shown below:

- ✓ Incubation period – 48 hours
- ✓ Inoculum concentration – 1.0 ml
- ✓ Carbon source – Glucose and Dextrose
- ✓ Nitrogen source – Tryptone and casein
- ✓ pH – 7.0
- ✓ Metal salts and Metal ions – 5% sodium chloride and Ferric chloride

Summary and Conclusion

5.0 SUMMARY AND CONCLUSION

At this time there are huge amounts of protein-rich by-product materials from seafood processing plants discarded without any attempt of recovery. Seafood industry generates large quantities of by-products as waste that includes viscera, shell, scales, fins and frame bones. Generally, shell portions of crab are removed during processing and these account for approximately 50% of the raw materials. Increasing production of inedible parts such as shells is causing environmental problems as a result of uncontrolled dumping. This alone contributes the good source of proteins including enzymes and fats. The large amount of crab waste can be used as a source for the isolation of enzymes, especially chitinase. It constitutes one of the most important groups of industrial enzyme which account for at least a quarter of the global enzyme production.

Bacteria and fungi from marine environments secrete different enzymes based on their habitat and their ecological functions. Especially these microorganisms from crab waste produce a best enzyme production such as chitinase. Microbial enzymes have several advantage over the enzymes derived from plant or animal sources by virtue of their great variety of catalytic activities, cheaper in cost, regular abundant supplies at even quantity and relatively more stability.

Enzymes are used in a variety of industrial processes to create an array of foods, cosmetics, nutraceuticals and pharmaceuticals. They offer advantages over chemical techniques including substrate specificity and elevated activity that allow better control of the production processes. However the use of enzymes in industrial applications requires their large scale production.

Thus, salient findings of the present study **“Optimization of Chitinase produced by Microorganisms Isolated from the Soil Degraded Crab Shell Waste”** are summarized as follows:

The present study was carried out using marine waste as soil degraded crab shell waste sample and soil sample collected from the disposable areas. The soil sample were air dried and the crab shell sample were homogenized, further it was serially diluted in sterile distilled water from 10^{-1} to 10^{-7} dilutions. 1 ml of the sample of each dilution was poured into tryptone soy agar medium and incubated at 37^0C for 24 hours. A well grown individual colony was assessed for chitinase production. These colonies were subcultured on nutrient agar plates. The plates were incubated at 37ºC for 24 hours and the colonies were observed.

A total of two samples were collected from which 7 bacterial strains were isolated. The bacterial strains were designated as CSS21 from soil sample, and CSW11, CSW21, CSW31, CSW32, CSW41 and CSW51 from crab shell sample. On the whole, 7 bacterial strains showed positive reactions in the plates containing colloidal chitin agar medium. From that, the positive plates indicate the colony which formed the hydrolysis zone after the incubation. The positive colonies were selected for further study.

The selected colonies were subcultured on nutrient agar slants. The microorganisms were identified by Gram staining and biochemical characterization and they belongs to the genus of *Bacillus*, *Neisseria meningitidis* and *E.coli*. From the above tests, *Bacillus* was isolated from both the soil and crab shell samples and *Neisseria meningitidis* and *E.coli* was isolated from only on the crab shell sample.

From the selected cultures to estimate protein content and chitinase activity based on the above test CSW31 have highest activity for chitinase production. So, CSW31 was selected for further study. The conditions needed for maximum biomass accumulation and enzyme production was carried out with different conditions, which includes incubation period, inoculum concentration, carbon source, nitrogen source, pH, metal salts and metal ions.

Incubation period was carried out ranging from 24 hours upto 96 hours. The incubation period for highest biomass was found to be at 96 hours

whereas for maximum enzyme production the optimum incubation period was 48 hours.

Inoculum concentration greatly influences the biomass accumulation and enzyme production. 0.5 ml to 5.0 ml range of inoculum concentration was used in optimization condition. The highest biomass was found to be at 2.0 ml and the enzyme production was maximum at 1.0 ml of inoculum.

To optimize the carbon sources for maximum biomass and enzyme production, different carbon sources were supplemented in the medium. From that, the biomass and enzyme production was higher in the presence of glucose and dextrose.

In case of nitrogen sources the biomass was higher in the presence of tryptone followed by casein. The amount of enzyme produced was estimated to be higher in the medium supplemented with tryptone and casein.

pH is the another important parameter for biomass and enzyme production. Different range of pH was carried out for the study from pH 5.0 to 10.0. The optimum pH for the biomass was found to be pH 7.0, the same pH gave the maximum enzyme production.

Optimization with metal salts was carried out and found that among various concentration of sodium chloride, 5% sodium chloride supported the maximum biomass and enzyme production followed presence of ferric chloride.

From that we can infer that the optimization of the culture growth of CSW31 was found to have higher biomass accumulation at 2.0 ml of inoculum concentration, incubation period at 96 hours which was maintained at pH 7.0.The best enzyme activity was observed when the medium was supplemented with dextrose and glucose as a carbon source, tryptone followed by casein as a nitrogen source and 5% sodium chloride and ferric chloride serve as metal salts and metal ions.

Hence we can infer from the above results the optimization of enzyme production culture of CSW31 was found to have higher enzyme activity at 1.0 ml of inoculum concentration, incubation period at 48 hours which was maintained at pH 7.0.The best enzyme activity was observed when the medium was supplemented with dextrose and glucose as a carbon source, tryptone and casein as a nitrogen source and 5% sodium chloride and ferric chloride serve as metal salts and metal ions.

Recommendations for the future studies:

- ✓ The study can be suggested to determine the temperature, amino acids and vitamins stability of the isolated organisms.
- ✓ Stability detection of the isolated organisms using the various substrates and its varying concentration.
- ✓ The study can be further analyzed for using fungal cultures.
- ✓ Large scale production can be carried out for isolation of chitinase from microorganisms.

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Appendices

APPENDIX I

Composition of tryptone soy agar medium

Ingredients	g/L
Pancreatic digest of casein	15
Papaic digest of soyabean meal	5
Sodium chloride	5
Agar	15
pH	7

Composition of nutrient agar

Ingredients	g/L
Peptone	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
Agar	15
pH	7.4

Composition of nutrient broth

Ingredients	g/L
Peptone	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
pH	7.4

Composition of basal medium

Ingredients	g/L
Disodium hydrogen phosphate	6
Potassium dihydrogen phosphate	3
Ammonium chloride	1
Sodium chloride	0.5
Yeast extract	0.05

Composition of colloidal chitin agar medium

(Kuddus and Ahmad, 2013)

Ingredients	g/L
Dipotassium hydrogen phosphate	3.5
Potassium dihydrogen phosphate	1.5
Magnesium sulphate	1
Tryptone	1
Yeast extract	1
Colloidal chitin	10
Agar	15

Preparation of colloidal chitin

(Kuddus and Ahmad, 2013)

Materials / Reagents

- Chitin powder
- Conc. HCl
- Distilled water

Procedure

- 10 g of chitin powder was slowly added in a beaker containing 150 ml of conc. HCl.
- The content were incubated for 60 minutes at 30ºC with vigorous stirring.
- Chitin was precipitated as a colloidal suspension by adding it slowly to water at 4ºC to 10ºC.
- The content were kept for over night at 4ºC .
- The content were kept in centrifugation at 6000 rpm for 30 minutes.
- The pellet was washed and repeated 3 times with distilled water until the pH of the suspension was 3.5.
- After that, the loose colloidal chitin was used as a substrate.

APPPENDIX II

Isolation of bacteria

Materials / Reagents

- Nutrient Agar medium (as described in Appendix I)
- Tryptone soy agar
- Soil sample
- Decomposed crab shell sample
- Sterile distilled water

Procedure

- 1g of soil and 1 ml of soil degraded crab shell homogenized sampled was mixed with 10 ml/9 ml of water separately to get a 10^{-1} dilution.
- Using the 10^{-1} dilution 1 ml of the sample was taken and serially diluted up to 10^{-7} dilution.
- From each dilution 100 µl of the samples was spread on to the tryptone soy agar plates.
- The plates were incubated at 37ºC for 24 hours in an inverted position and the colonies were observed.
- Well grown individual colonies were assessed for chitinase production. These colonies were subcultured on nutrient agar plates.
- The plates were incubated at 37ºC for 24 hours and the colonies were observed.
- The individual colonies were isolated.

APPENDIX III

Screening of chitinase producing bacteria

(Kuddus and Ahmad, 2013)

Materials / Reagents

- Colloidal chitin agar medium

Procedure

- The isolated bacterial colonies were inoculated in a colloidal chitin agar medium.
- The plates were incubated at 37ºC for 48 hours.
- After the incubation period, a larger hydrolysis zone was observed.
- The cultures were centrifuged at 10000 rpm for 15 minutes at 4ºC.
- Then the crude was used as enzyme for chitinase assay.

APPENDIX IV

Gram Staining

(Sundarajan, 1995)

Principle

The gram – positive bacteria will retain the crystal violet and appear deep violet in colour. The gram negative bacteria lose crystal violet on decolourization and are counter stained by the safranin and appear red in colour. Iodine solution is used as a mordant that fixes the primary stain in or on a substrate by combining with the dye to form an insoluble compound – mordant, for the first stain. The exact mechanism of action is thought to be associated with the structure and composition of the cell wall.

The cell walls of Gram – negative bacteria are thinner than that of Gram – positive bacteria and contain higher percentage of lipid content. During the staining of Gram – negative bacteria, the alcohol treatment extracts the lipid. This results in increased porosity and permeability of the cell. The crystal – violet iodine (CV - I) complex, thus can be extracted and the Gram – negative bacteria is decolourized. The cells subsequently take up the colour of the counter stain safranin.

The cell walls of Gram – positive bacteria, with lower lipid content, become dehydrated during alcohol treatment. The pore size decreased, permeability is reduced and the CV – I complex cannot be extracted. Therefore, the Gram – positive cells remain purple – violet.

Materials / Reagents

i. Solution A
   - Crystal violet - 2g
   - Ethanol - 20ml

ii. Solution B
   - Ammonium oxalate - 0.8g
   - Distilled water - 80ml

Mix solution A and B to obtain the crystal violet staining reagent. Store for 24 hours and filter through paper before using.

iii. Mordant

- Iodine - 1g
- Potassium iodide - 2g
- Distilled water - 300ml

Grind the iodine and potassium iodide in a motor and add water slowly with continuous grinding until iodine is dissolved. Store in amber coloured bottles.

iv. Decolorizing agent

- Ethanol - 95%

v. Counterstain

- Safranin - (2.5% in 95% ethanol)
- Distilled water - 100ml

Procedure

- Air dry or heat fix the bacterial smear. Flood the slide with crystal violet staining reagent for 1 minute.
- Wash the smear in a gentle and direct stream of tap water for 2 seconds.
- Flood the slide with iodine mordant for 1 minute.
- Wash the smear in a gentle and direct stream of tap water for 2 seconds.
- Blot the smear dry with absorbent paper.
- Immerse the smear for 2 minutes with counter stain.
- Wash the smear in a gentle and indirect stream of water until no colour appears in the wash waterblot the smear dry with the absorbent paper.
- Examine under the microscope.

APPENDIX V

Biochemical Tests

Indole Test

(Kannan, 2002)

Principle

Tryptophan is oxidized with the tryptophanase resulting in the formation of indole pyruvic acid and ammonia. The indole test is carried out by inoculating a bacterium into peptone broth and the indole produced during this reaction is indicated by adding Kovac's reagent (p-dimethyl aminobenzaldehyde) which produces a cherry red colour.

Materials / Reagents

i. 1% peptone broth

- Peptone - 10g/L
- NaCl - 5g/L

ii. Kovac's reagent / Ehrlisch reagent

5 g of p-dimethyl aminobenzaldehyde was dissolved in 75ml of amyl alcohol. 25 ml of conc. HCl was added to it and mixed well.

Procedure

- Two tubes of peptone broth were prepared and sterilized in an autoclave at 15 lbs for 15 minutes.
- One of the tubes of peptone broth was inoculated with the test sample and the second tube was kept as an uninoculated control.
- The tubes were inoculated at 37ºC for 48 hours.
- After incubation period, 1ml Kovac's reagent was added to the tube containing test sample and control tube.
- The tubes were gently shaken with intervals of about 10 -15 minutes.
- The tubes were allowed to stand to permit the reagent to rise to the top. Positive reaction was detected by the formation of cherry red colour.

Methyl Red Test

(Kannan, 2002)

Principle

This is to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in colour at a pH of 4.4 or less.

Materials / Reagents

i. Methyl Red – Voges Proskauer Broth

- Peptone - 5g/L
- Dextrose - 5g/L
- Dipotassium hydrogen phosphate - 5g/L

ii. Methyl Red reagent

- Methyl red - 100mg
- Ethanol - 300ml
- Distilled water - 200ml

Transferred methyl red to ethanol, dissolved well, added water and then filtered.

Procedure

- 5ml of MR-VP broth was added into the two tubes, one marked as test sample and the other as control.
- The broth was inoculated with the test sample and control tube was kept uninoculated.
- The tubes were incubated for 24 hours at 37ºC.
- After incubation, 5 drops of methyl red indicator was added to each tubes. Positive reaction was indicated by formation of red colour.

Voges Proskauer Test

(Kannan, 2002)

Principle

This test is to determine the capacity of some organism to ferment carbohydrates with the production of non – acidic or neutral end products such as acetyl methyl caribinol or its reduction products 2,3 butylene glycol. These products are produced from the organic acids that results from glucose metabolism which is characteristic of *Enterobacter aerogens*.

Materials / Reagents

i. Methyl Red – Voges Proskauer Broth

- Peptone - 5g/L
- Dextrose - 5g/L
- Dipotassium hydrogen phosphate - 5g/L

ii. 40% potassium hydroxide

iii. 3% α – naphthol

Procedure

- Inoculate a test culture in the MR – VP medium.
- Incubate at 37ºC for 48 hours.
- After 48 hours of incubation, add 1ml of 40% potassium hydroxide and 3% α – naphthol.
- A positive reaction is indicated by the development of a pink colour in 2 – 5 minutes becoming crimson in 30 minutes.

Citrate Utilization Test

(Kannan, 2002)

Principle

In the absence of glucose or lactose, some microorganisms use citrate as the carbon source which depends on the presence of citrate permease. Citrate is acted upon by the enzyme citrase which produces oxaloacetic acid and acetate. These are then enzymatically converted to pyruvic acid and carbondioxide. During this reaction, the medium becomes alkaline as the carbondioxide combines with sodium and water to form sodium carbonate, an alkaline product. The sodium carbonate changes bromothymol blue from green to deep prussian blue.

Materials / Reagents

i. Simmons citrate agar

- Sodium chloride - 5g
- Magenesium sulphate - 0.2g
- Ammonium dihydrogen phosphate - 1g
- Potassium dihydrogen phosphate - 1g
- Sodium citrate - 5g
- Distilled water - 1000ml
- Agar - 20g
- Bromothymol blue (0.2%) - 40ml
- pH - 6.8

Procedure

- Inoculate the test organism on agar slopes.
- Incubate at 37ºC for 96 hours.
- Those cultures that show pH shift of the medium to alkaline conditions should be considered positive.

Oxidase Test

(Kannan, 2002)

Principle

To determine the presence of oxidase enzymes, the reagent (impregnated into strips of filter paper), which contains tetramethyl-p-phenylenediamine, serves as an alternate substrate for the cytochrome oxidase reaction. In the reduced state the reagent is colourless but when oxidized it becomes purple.

Materials / Reagents

i. 1% Tetramethyl-p-phenylenediamine dihydrochloride

ii. Filter paper disc

iii. Sterile slide

iv. Sterile glass rod

Procedure

- The filter paper disc impregnated with oxidase reagent was placed aseptically on a clean sterile slide.
- With the help of sterile glass rod, a small amount of culture was transferred to one disc.
- The colour change of the disc was examined.

Catalase Test

(Kannan, 2002)

Principle

Microorganisms produce hydrogen – per – oxide during respiration and in some instances, an extremely toxic superoxide. Organism capable of producing catalase or peroxidase rapidly degrade hydrogen peroxide. Anaerobes are unable to synthesize catalase, peroxidase or superoxidase dismutase.

Materials / Reagents

i. Nutrient agar

ii. 3% Hydrogen peroxide

Procedure

- Inoculate a Nutrient agar slant with the test culture or any other medium lacking blood.
- Incubate at 37ºC for 24 hours. Following incubation, pipetted out 1ml of 3% hydrogen peroxide down the slant.
- Examine immediately and after 5 minutes for the evolution of bubbles which indicates a positive test.

Starch Hydrolysis Test

(Kannan, 2002)

Principle

Starch is a linear polymer of glucose molecule linked together by glycosidic bonds. Starch as such cannot be transported into the cell for energy production, because of its high molecular weight. To assimilate starch for energy and catabolic reactions, it must be degraded into basic glucose units by starch hydrolyzing enzymes. These enzymes are secreted by microorganisms into the medium which degrade starch primarily to glucose. The resulting low molecular weight soluble glucose molecules are now able to pass into then cell for energy production via., glycolysis.

Materials / Reagents

i. Starch agar

- Peptone - 5g
- Beef extract - 3g
- Soluble starch - 2g
- Agar - 15g
- pH - 7.0

ii. Iodine solution

Procedure

- Streak the plates with suitable culture maintaining at least two plates for each culture and one uninoculated as the control.
- Incubate the plates for 48 hours at 37ºC in an inverted position.
- Flood the surface of the plate with iodine solution with a dropper for 30 seconds.
- Pour off the excess iodine solution.
- Examine the plates for starch hydrolysis around the line of growth i.e the colour change of the medium.
- A clear zone surrounding the microbial growth indicates a positive reaction.
- A negative reaction is indicated by a dark colouration of the medium.

Carbohydrate Fermentation Test

(Kannan, 2002)

Principle

In bacteria, bio oxidation reactions are very important as they help provide energy either by oxidation of organic substances or by fermentation. In the earlier case, by utilizing organic compounds as electrons donors with oxygen as the ultimate acceptor, bacteria produce carbondioxide and water. The ability to utilize free oxygen is due to Cytochrome enzyme system and the process is called respiration while fermentative organisms utilize organic compounds but lack a cytochrome system and produce carbondioxide and water besides a complex end product such as alcohol, acid, or aldehyde. The acid, alkali or gas production results in a visible change in the inoculated broth due to the presence of suitable indicator. The fermentation is carried out in a fermentation tube which is a culture tube containing a Durham tube for detection of gas production.

Materials / Reagents

i. Broth or single colony cultures of the given bacteria
ii. Fermentation broth of glucose
iii. Inoculation loop
iv. Test tubes
v. Durham tube
vi. Medium
    - Peptone (Trypticase) - 10.g
    - Glucose - 5g
    - Phenol red - 0.018g
    - Distilled water - 1000ml
    - pH - 7.3

Procedure

- Fermentation broth was prepared with sugar as the carbohydrate source.
- Added approximately 10ml in the test tubes maintain three tubes for each given culture and one tube as uninoculated control.
- Inverted Durham's tube into all of the test tubes, plugged it with cotton and autoclaved at 15lbs pressure for 20 minutes.
- Inoculated each culture in their respective tubes except in the control.
- Incubate all the tubes at 37ºC for 24 – 48 hours.
- Observe the reactions that develop in the fermentation medium by comparing with the control i.e change in colour from red to yellow (due to acid production) and / or appearance of bubbles (due to gas production).

Triple Sugar Iron Test

(Kannan, 2002)

Principle

Triple Sugar Iron (TSI) test is used to differentiate different group of the *Enterobacteriaceae* according to their ability to ferment lactose, sucrose and glucose and the production of hydrogen sulphide. The fermentation reaction of the sugars will help to distinguish between *Enterobacteriaceae* from other gram negative intestinal bacilli.

Materials / Reagents

i. TSI slants
   - Lactose - 1%
   - Sucrose - 1%
   - Glucose - 0.1%
   - Phenol red

Procedure

- Inoculate a slant of TSI agar using a straight needle.
- Incubate at 37ºC for 18 – 24 hours.
- The results after incubation will be of three kinds i.e sugar fermentation, gas production and hydrogen production.

Nitrate Reduction Test

(Kannan, 1996)

Principle

Certain bacteria use nitrates in the places of oxygen as an external terminal acceptor. Nitrate can easily be reduced to nitrite by nitrate reductase. In aerobic bacteria, oxygen is first used to prevent nitrate reduction and utilize nitrate. The nitrite may further give rise to nitrogen, ammonia an oxide.

Materials / Reagents

i. Nitrate broth

ii. 1% α – Napthalamine reagent

iii. 1% sufanilic acid

Procedure

- Nitrate broth was sterilized. And inoculate the organism into it.
- Incubated the content at 37ºC for 18 – 24 hours. After incubation, 0.5ml of 1% α – Napthalamine reagent was added.
- Followed by 0.5ml of 1% sufanilic acid was added.Red coloration indicates the positive result.
- Absence of red colour indicates the negative result.

Urea Hydrolysis Test

(Kannan, 1996)

Principle

Urea is a waste product of nitrogenous material excreted out by animals. Some degrade the nitrogen and carbon bond in urea to form carbon dioxide in the presence of a hydrolytic enzyme urease. The carbon dioxide reacts to form ammonium carbonate, an alkaline end product, an increases the pH of the medium. This can be detected by incorporating a pH indicator in the medium, which changes the colour during alkaline conditions.

Materials / Reagents

i. Christensen's Urea Agar Medium

Procedure

- Sterile Christensen's Urea Agar tubes were prepared.
- The tubes were inoculated with the bacterial culture.
- Incubate the tubes at 37ºC for 24 – 48 hours.
- Urease positive tubes are deep pink in colour.
- No colour change indicates negative result.

APPENDIX VI

Quantitative Assay for Chitinase

(Aida and Taghreed, 2014)

Principle

Chitinase converts chitin into chitobiose in presence of water which then leaves the product N – acetyl – D – glucosamine with the action of the enzyme β – N acetyl glucosaminidase.

Materials / Reagents

i. Crude enzyme
ii. 1% Colloidal chitin
iii. 0.02 M Phosphate buffer (pH – 7.0)
iv. Distilled water
v. Dinitrosalicylic acid reagent

Procedure

- 0.5 ml of crude enzyme was added to the mixture of 0.5 ml of 1% Colloidal chitin suspended in 0.02 M Phosphate buffer (pH – 7.0).
- Incubate the content at 40ºC for 30 minutes.
- After incubation 1.0 ml of dinitrosalicylic acid reagent added into it for stop the reaction.
- The reaction mixture were kept in a boiling water bath for 10 minutes at 100□C.
- After cooling, the content were centrifugation at 8000 rpm for 10 minutes.
- The absorbance of the mixture was measured at 530nm.

Calcualtion

One unit of the chitinase activity was defined as the amount of enzyme which yields 1µmol of reducing sugar as N – acetyl – D – glucosamine (GlcNAc) equivalent per minute.

APPENDIX VII

Estimation of Protein

(Lowry *et al*., 1951)

Principle

The blue colour developed by the reduction of phosphomolybdic phosphotungstic components in the Folin – Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein, plus the colours developed by the biuret reaction of the protein with alkaline cupric tartarate are measured in Lowry's method.

Materials / Reagents

i. Solution A
   - 1% Copper sulphate

ii. Solution B
   - 2% Sodium potassium tartarate

iii. Solution C

- 2% Sodium carbonate in 0.1 N NaOH

iv. Solution D

- Mixed just before use, 1ml of solution A, 1ml of solution B and 100ml of solution C.

v. Solution E

- 1N Folin Ciocalteau reagent (Mixed equal volumes of commercially available reagent and distilled water just prior to use). Stored protected from light.

vi. Standard BSA

- 50mg BSA in 50ml of 0.1 N NaOH. Diluted 1:10 for working standard.

Procedure

- Aliquots of standard protein solution (0 -1000µg) were taken. The enzyme sample were made upto 1ml with 0.1 N NaOH. Shook well to treat the protein with alkali.
- Added 1ml of solution D, mixed well.
- Incubated the tubes at 37ºC for 3 minutes.
- After the incubation, added 0.1ml of solution E to each tube and mixed well.
- Then incubated the content at 37ºC for 3 minutes.
- Read the colour developed at 670nm against a reagent blank.

APPENDIX VIII

Determination of Biomass

Principle

Bacterial population (cell mass) or amount of growth can be determined by measuring the turbidity or optical density of a broth culture. The light transmission is less when there is more turbidity. Since turbidity is directly proportional to the number of cells, this property is used as an

indicator of bacterial concentration in a sample. Turbidity is quantified with a colorimeter that measures the amount of light energy. The light energy transmitted or absorbed directly through suspension is measured at 600nm as the percentage of transmission (%T) and is directly proportional to the cell mass concentration.

Materials / Reagents

i. Nutrient broth

ii. Bacterial culture

Procedure

- Sterile nutrient broth was prepared in a conical flask.
- The flasks were inoculated using bacterial culture.
- The content was incubated at 37ºC for 24 hours.
- After incubation, to determine the biomass, 3ml of the fermented broth was taken and the optical density was read at 600nm