APPLICATION OF FERMENTATION TECHNIQUES IN THE UTILIZATION OF PRAWN SHELL WASTE

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In partial fulfillment of the requirements for the degree of

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IN MICROBIOLOGY

By

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There is nothing more powerful than an idea whose time has come...

– Victor Hugo

Dedicated...

To My Beloved Papa...

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CERTIFICATE

This is to certify that the Ph.D. thesis entitled "APPLICATION OF FERMENTATION TECHNIQUES IN THE UTILIZATION OF PRAWN SHELL WASTE" embodies the original research work conducted by Mrs. Sini. T.K. (Reg. No. 2611), under my guidance from January 2003 to Novermber 2007. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associate ship, fellowship or any other similar titles of this or in any other university or institution. She has also passed the PhD qualifying examination of the Cochin University of Science and Technology, held in October 2004.

Dr. P.T. Mathew
Principal Scientist,
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Dated: 11/12/2007

DECLARATION

I, SINI.T.K., do hereby declare that the Thesis entitled **"APPLICATION OF FERMENTATION TECHNIQUES IN THE UTILIZATION OF PRAWN SHELL WASTE"** is a genuine record of bonafide research carried out by me under the supervision of Dr. P.T.Mathew, Principal Scientist, Fish Processing Division, Central Institute of Fisheries Technology, Cochin-682 029. This work has not previously formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or any other university or Institution.

Cochin-29,
12-12-2007

SINI.T.K.

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Sini. T.K.

ABBREVIATIONS

AR	-	Analar
BSA	-	Bovine serum albumin
°C	-	Degree celsium
EDTA	-	Ethylene diamine tetra acetic acid
Fig.	-	Figure
FFA	-	Free fatty acid
g	-	grams
HCl	-	Hydrochloric acid
H_2SO_4	-	Sulphuric acid
NaOH	-	Sodium hydroxide
h	-	Hour
Kg	-	Kilogram
KOH	-	Potassium hydroxide
M	-	Molar
mM	-	Millimolar.
mg	-	Milligram
Mm	-	Millil-molar.
mm	-	Millimeter
N	-	Normal
µ moles	-	Micro molesmin
Min	-	Minute
ml	-	Militer
SD	-	Standard division
v/v	-	Volume/volume
w/v	-	Weight/volume
w/w	-	Weight/weight

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ABSTRACT

The present study aimed at the utlisation of microbial organisms for the production of good quality chitin and chitosan. The three strains used for the study were *Lactobacillus plantarum*, *Lactobacililus brevis* and *Bacillus subtilis.* These strains were selected on the basis of their acid producing ability to reduce the pH of the fermenting substrates to prevent spoilage and thus caused demineralisation of the shell. Besides, the proteolytic enzymes in these strains acted on proteinaceous covering of shrimp and thus caused deprotenisation of shrimp shell waste. Thus the two processes involved in chitin production can be affected to certain extent using bacterial fermentation of shrimp shell.

Optimization parameters like fermentation period, quantity of inoculum, type of sugar, concentration of sugar etc. for fermentation with three different strains were studied. For these, parameters like pH, Total titrable acidity (TTA), changes in sugar concentration, changes in microbial count, sensory changes etc. were studied.

Fermentation study with *Lactobacillus plantarum* was continued with 20% w/v jaggery broth for 15 days. The inoculum prepared yielded a cell concentration of approximately 10^8 CFU/ml. In the present study, lactic acid and dilute hydrochloric acid were used for initial pH adjustment because; without adjusting the initial pH, it took more than 5 hours for the lactic acid bacteria to convert glucose to lactic acid and during this delay spoilage occurred due to putrefying enzymes active at neutral or higher pH. During the fermentation study, pH first decreased in correspondence with increase in TTA values. This showed a clear indication of acid production by the strain. This trend continued till their proteolytic activity showed an increasing trend. When the available sugar source started depleting, proteolytic activity also decreased and pH increased. This was clearly reflected in the sensory evaluation results. Lactic acid treated samples showed greater extent of demineralization and deprotenisation at the end of fermentation study than hydrochloric acid treated samples. It can be due to the effect of strong hydrochloric acid on the initial microbial count, which directly affects the fermentation process. At the end of fermentation, about 76.5% of ash

was removed in lactic acid treated samples and 71.8% in hydrochloric acid treated samples; 72.8% of proteins in lactic acid treated samples and 70.6% in hydrochloric acid treated samples.

The residual protein and ash in the fermented residue were reduced to permissible limit by treatment with 0.8N HCl and 1M NaOH. Characteristics of chitin like chitin content, ash content, protein content, % of N- acetylation etc. were studied. Quality characteristics like viscosity, degree of deacetylation and molecular weight of chitosan prepared were also compared. The chitosan samples prepared from lactic acid treated showed high viscosity than HCl treated samples. But degree of deacetylation is more in HCl treated samples than lactic acid treated ones. Characteristics of protein liquor obtained like its biogenic composition, amino acid composition, total volatile base nitrogen, alpha amino nitrogen etc. also were studied to find out its suitability as animal feed supplement.

Optimization of fermentation parameters for *Lactobacillus brevis* fermentation study was also conducted and parameters were standardized. Then detailed fermentation study was done in 20%w/v jaggery broth for 17 days. Also the effect of two different acid treatments (mild HCl and lactic acid) used for initial pH adjustment on chitin production were also studied. In this study also trend of changes in pH. changes in sugar concentration ,microbial count changes were similar to *Lactobacillus plantarum* studies. At the end of fermentation, residual protein in the samples were only 32.48% in HCl treated samples and 31.85% in lactic acid treated samples. The residual ash content was about 33.68% in HCl treated ones and 32.52% in lactic acid treated ones. The fermented residue was converted to chitin with good characteristics by treatment with 1.2MNaOH and 1NHCl.Characteristics of chitin samples prepared were studied and extent of N-acetylation was about 84% in HCl treated chitin and 85%in lactic acid treated ones assessed from FTIR spectrum. Chitosan was prepared from these samples by usual chemical method and its extent of solubility, degree of deacetylation, viscosity and molecular weight etc were studied. The values of viscosity and molecular weight of the samples prepared were comparatively less than the chitosan prepared by *Lactobacillus plantarum* fermentation. Characteristics of

protein liquor obtained were analyzed to determine its quality and is suitability as animal feed supplement.

Another strain used for the study was *Bacillus subtilis* and fermentation was carried out in 20%w/v jaggery broth for 15 days. It was found that Bacillus subtilis was more efficient than other Lactobacillus species for deprotenisation and demineralization. This was mainly due to the difference in the proteolytic nature of the strains. About 84% of protein and 72% of ash were removed at the end of fermentation. Considering the statistical significance ($P<0.05$) in the extent of demineralization and deproteinisation, we have taken 0.8N HCl for the demineralization study and 0.6M NaOH for deprotenisation study. Properties of chitin and chitosan prepared were analyzed and studied.

1. INTRODUCTION

Prevalence of fast food stuffs and ready to eat food products have become a part of the swift running lifestyle. Eventhough these eatables are more appealing in taste; they may lack essential nutrients. Here comes the beneficiary of our traditional food habit. Seafood holds a very unique position in our traditional menu. It is peculiar with high nutritional value and easy digestibility. Export of processed and frozen shrimp products is the backbone of seafood industries. Shrimp industry is a rapidly growing industry in India and all over the world. But major curse of seafood industry is the large amount of waste materials, which are highly perishable in nature as they are quickly colonised by spoilage organism and can rapidly be transferred into a public health hazard (Sini *et al.*, 2005). In South East Asia, more than two million metric tonnes of wastes was produced per year (Hussain, 2003). In India, Shrimp waste constitutes more than one lakh tonnes per year (Philip & Nair, 2006). The shrimp waste which is constituted by its head, thorax, claws and its shell contributes to 45% of its weight (Zakaria *et al.*, 1998). Shrimp shell is a rich source of many valuable products like protein, mucopolysaccharide, pigments, flavour compounds etc. (Ornum, 1992). Effective utilisation of these products creates a high economic value to waste substances. Shrimp waste contains about 10-20% calcium, 30-40% protein and 8-10% chitin (Legarreta *et al.*, 1996). Proteins, the major component extracted from the shrimp shell can be used as an animal feed supplement (Meyers and Benjamin, 1987). Astaxanthin is utilized as pigment in salmonid feeds (Guillou *et al.*, 1995). Chitin is the second most abundant biopolymer next to cellulose (Yang *et al.*, 2000). Chitin and its derivatives like chitosan, carboxymethyl chitin etc. have found immense applications in biomedical field, biotechnology, food industry, textiles, paper industry etc. (Santhosh *et al.*, 2006). The variety in applications of these products in different fields depends on its physicochemical properties like degree of deacetylation, viscosity, molecular weight etc.

Traditional methods for the commercial preparation of chitin from shrimp shell involve alternate hydrochloric acid and sodium hydroxide treatment stages to remove calcium carbonate and proteins, respectively, followed by a bleaching stage with chemical reagents like hydrogen peroxide to obtain a colourless product (Bautista *et al.*, 2001). Disposal of the wastewater from these treatments is a serious environmental problem. These chemical treatment may cause partial deacetylation of chitin and hydrolysis of the polymer, leading to inconsistent physiological properties in the end product (Brine and Austin, 1981).And the cost of the process is also very high and protein liquor obtained cannot be used as animal feed.

To overcome the problems of chemical treatment, studies have been conducted with proteolytic enzymes like pepsin, chymotrypsin, trypsin, papain etc to remove protein and thus to produce chitin (Gagne and Simpson 1993; Broussignac, 1968). Many authors tried microorganisms to deprotenise and demineralise the shell waste to produce chitin (Bustos and Micheal,1994a, Cira *et al.*, 2002; Shirai *et al.*, 2001; Teng *et al.*, 2001).

Lactic acid fermentation might well offer a commercial route for the recovery of chitin and other products from shellfish wastes (Hall and Reid, 1994; Hall and De Silva,1992). During fermentation with lactic acid bacteria, it produces lactic acid and cause rapid acidification of the raw material (Caplice and Fitzgerald, 1999; Ray, 1992. Wood 1997; Wood and Holzapfel, 1995). The acid produced was responsible for the demineralization process. The proteins are removed by the action of proteolytic enzymes of lactic acid bacteria (Law and Haandrikman, 1997). Also their production of acetic acid, ethanol, aroma compounds, bacteriocins and exopolysaccharides and several enzymes enhance shelf life, improved texture and contribute to the pleasant sensory profit of the end products (Champomier-Verges *et al.*, 2002). As lactic acid bacteria are considered as food grade organism, the protein liquor obtained by fermentation can be effectively utilized as animal feed ingredient.

The most commonly used lactic acid bacteria are *Lactobacillus plantarum*, *Lactobacillus acidophilus* ,*Pedicoccus acidilactici* ,*Pedicoccus pentosus*,

Lactobacillus pentosus, *Lactobacillus paracasei* etc.The protease enzymes of *Bacillus subtilis*, *Bacillus firmus* are by far the most important group of Bacillus species exploited for degradation of proteinaeous waste into useful biomass (Atalo and Gashe, 1993, Venugopal *et al.*, 1989).

Several studies reported the production of chitin by lactic acid fermentation of shrimp waste (Zakaria *et al.*, 1998; Rao *et al.*, 2000; Cira *et al.*, 2002; Fagbenro and Bello-Olusoji, 1997). This method retains the physicochemical properties of chitin to a greater extent since there is no drastic acid or alkali treatment. Chitin is converted to chitosan by the N-deacetylation.

2. REVIEW OF LITERATURE

2.1. Seafood

Seafood is divinely rich by virtue of its high nutritional value. Export of processed sea food items bags good revenue for the country. Seafood processing industry in India is contributing tonnes and tonnes of waste materials and amongst them shrimp waste contributes more than one lakh tonnes every year (Philip and Nair, 2006). The discharge of these waste material is a serious environmental problem, as they are quickly colonized by spoilage organisms (Sini *et al.*, 2005). The efficient utilization of these wastes yields high economic value, as these wastes are rich in proteins, mucopolysaccharides like chitin, pigments, flavour compunds etc. (Zakaria *et al.*, 1998). Pollution created by high volume shrimp production requires both the application of a traditional preservation method, such as lactic ensilation, and the feasibility of by-products recovery (Cira *et al.*, 2002).

2.1.1. Chitin and Chitosan

Chitin, the second most abundant biopolymer next to cellulose (Yang *et al.*, 2000) is present in the shell of crustaceans, the exoskeletons of insects and cell walls of fungi and some algae (Fig. 2.1.). It is white, hard and inelastic in nature. This compound was first isolated by Braconnot in 1811 from mushrooms and was named "fungine" (Madhavan, 1992). The annual global yield of chitin is assumed to be 1 to 100 billion metric tons, making chitin the second most abundant polysaccharide on the earth. Chitinous substances, accounting for 10-55% in dry weight, are contained in shrimp, crab, cuttlefish, squid, oyster etc. (Rattanakit *et. al.*, 2002)

Chitin is a polymer of β-(1-4) N-acetyl-D-glucosamine (Fig. 2.2.). Chitin occurs in three polymorphic forms, which differ in the arrangement of molecular chains within the crystal cell. α- chitin is the tightly compacted, most crystalline

Fig. 2.1. Chitin, Chitosan and their raw materials

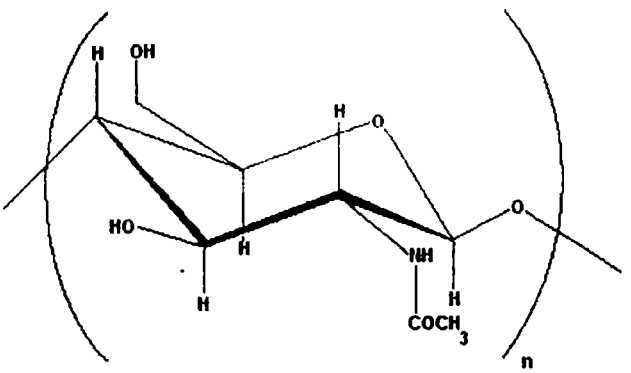
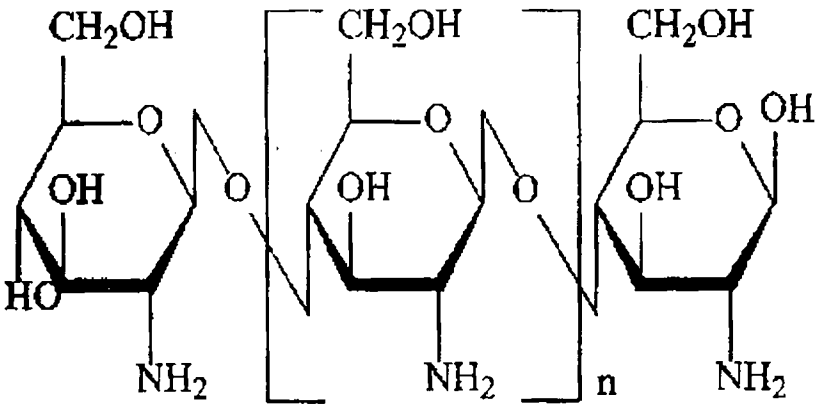


Fig. 2.2. Structure of Chitin

polymorphic form where the chains are arranged in an anti-parallel fashion. β-chitin is the form where the chains are parallel and γ-chitin is the form where two chains are "up" to every one "down" (Vaaje-Kolstad *et al.*, 2005). Deacetylation of chitin with strong alkali yields chitosan (Fig. 2.3.), a polymer of β-(1-4) D-glucosamine (Santhosh *et al.*, 2006).

Chitin is highly hydrophobic and is insoluble in water and most organic solvents. It is soluble in hexafluoro isopropanol, hexafluoro acetone, chloroalcohols in conjunction with aqueous solutions of mineral acids and dimethyl acetamide containing 5% lithium chloride. The acetyl group connected to an amine group in the C2 position on the glucan ring may be removed by enzymatic or chemical hydrolysis in caustic soda at elevated temperatures, producing a deacetylated form exposing free amino groups at some of the C2 positions. When the fraction of acetylated amine groups (FA) is lower than 0.35-0.40, the co-polymer of D-glucosamine ($GlcNH_2$) and N-acetyl-D-glucosamine (GlcNAc) formed referred to as chitosan (Averbach, 1975). Chitosan is soluble in weak acids and insoluble at neutral pH (Benjakul *et al.*, 2001). The chitin polymers are embedded in a protein structure, which may be calcified with salts forming a hard shell structure (Rao *et al.*, 2000). Chitin and chitosan are now produced commercially in Japan, U.S.A., India, Poland, Norway and Australia. Chitin and chitosan are of commercial interest due to their high percentage of nitrogen (6.89%) compared to synthetically substituted cellulose (1.25%) (Jayakumar *et al.*, 2006).

Chitin and its derivatives hold great economic value because of their versatile biological activities and chemical applications (Andrade *et al.*, 2003). The unique properties of chitin and its derivative, chitosan include solubility behaviour in various media, solution viscosity, polyelectrolyte behaviour, polyoxysalt formation, ability to form films, chelate metal ions, and optical and structural characteristics (Madhavan, 1992). The ability of chitosan to wrap the solid particles suspended in liquid and agglomerate makes it suitable in clarification and purification application in waste water treatment plants and in food industry (Prabaharan and Mano, 2006; Crini, 2006; Muzzarelli, 1996; Dodane and Vilivalam, 1998). Chitosan is used in chromatography because of



Chitosan

Fig. 2. 3. Structure of chitosan

the presence of free amino and hydroxyl groups. Chitosan is used as good sizing agent in textile industry. The chelating ability, adhesive property and ionic bond forming characteristics of chitosan find potential application in textiles. Fabrics sized with chitosan have good stiffness, improved dye uptake, added lusture and improved laundering resistance. Its high molecular weight, polycationic nature, film forming and hydrogen bonding ability make it suitable in paper industry. Chitin and chitosan can be used for the production of fibers and films and chitin films are stronger than chitosan films (Macleod *et al.*, 1999). Chitosan has important applications in photography due to its resistance to abrasion, optical characteristics, film forming ability and behaviour with silver complexes. In food industry, its main application is that it can be used as hyolipedemic and hypocholersterolemic agent (Vaaje-Kolstad *et al.*, 2005). Chitin and its derivatives have potential application in agriculture for various uses, such as, in germination and culturing, to enhance self protection against pathogenic organisms in plants and suppress them in soil, to induce chitinase activity and proteinase inhibitor synthesis, for antivirous activity, in encapsulation of fertilizers, in liquid fertilizers and in controlled release of herbicides. (Banos *et al.*, 2006; Hirano, 1996; Illum, 1998).

Chitin and chitosan have got immense applications in medical field. It helps in controlled release of drug, immobilization of enzymes, act as dialysis membrane etc. It acts as bacteriostatic agent, haemostatic agent, spermicide, anticholesterimic agent, anticoagulant, tissue regenerating agent and wound healing agent (Jayakumar *et al.*, 2006; Martino *et al.*, 2005; Kweon *et al.*, 2003; Mi *et al.*, 2003). It has got application in ophthalmology by making contact lens and in dentistry as wound healing agent and promotes fibrin formation.

Protein extracted from the shrimp shell waste has been proved to be an excellent animal feed supplement (Meyers and Benjamin, 1987). Shrimp waste can be effectively converted into silage through an environment-friendly safe technology by using organic acids alone and in combination with Lactobacillus (Raa and Gildberg, 1982; Dapkevic us *et al.*, 1998; Haard *et al.*, 1985)

The traditional methods of chitin production involve the use of strong alkalis and acids, making this process ecologically aggressive and a source of pollution (Bautista *et al.*, 2001). It also promoted a certain degree of depolymerization, reducing chitin quality (Simpson *et al.*, 1994; Healy *et al.*, 1994). The acid/alkali process renders the protein component useless, which otherwise can be used as fish feed.

To tide over the shortcomings of chemical treatment, deproteinisation trials using microorganisms (Shimahara and Takiguchi, 1988, Bustos and Micheal, 1994; Wang and Chio, 1998; Cira *et al.*, 2002; Shirai *et al.*, 2001) and proteolytic enzymes (Gagne and Simpson,1993; Broussignac,1968) were reported. Demineralisation also takes place during bacterial fermentation. During fermentation with microbes, deprotenisation takes place by the activity of proteases in the microorganisms and demineralization by the acid produced by the microorganisms (Rao *et al.*, 2000).

Broussignac (1968) demonstrated that the use of papain, trypsin, or pepsin produced chitin with as little deacetylation as possible. Bustos and Healy (1994) demonstrated that chitin obtained by the deproteinisation of shrimp shell waste with various proteolytic microorganisms including *Pseudomonas maltophilia*, *Bacillus subtilis*, *Streptococcus faecium*, *Pediococcus pentosaseus* and *Aspergillus oryzae* had higher molecular weights compared to chemically prepared shellfish chitin. Teng *et al.* (2001) produced chitin from shrimp shell using fungal mycelia produced chitin with high molecular weight (10^5 dalton range) and better degree of N-acetylation. Both deproteinisation and demineralization of shrimp shell obtained on fermentation with *Asperigillus niger*. Treatment of acid and alkali for the preparation of chitin cause the depolymerisation of chitin and deacetylation of chitin (Simpson *et al.* 1994; Brine and Austin, 1981). According to Coughlin (1990), there is great demand for partially purified chitin, and in that case chitin prepared out of fermentation can be used. Crayfish chitin was isolated by Bautista *et al.* (2001) by batch fed fermentation using *Lactobacillus pentosus*. Many authors reported mild acid and alkali treatment of raw chitin obtained by fermentation to produce purified chitin (Bautista *et al.*, 2001; Cira *et al.*, 2002). They standardized different

concentrations of HCl and sodium hydroxide and the concentration depends on the residual protein and ash content.

2. 2. Fermentation

Food biotechnology, as we know it today, is rooted in the development of fermented foods during 6000 to 12000 years of man's cultural history. Inhabitants of many tropical and subtropical regions rely on fermentation as a mean of preserving and safeguarding their food and also for their typical sensory characteristics (Holzapfel *et al.* 1995). Traditional fermentation depends on naturally occurring microorganism in the substrate or materials involved in the process such as tools, equipment or human handling (Steinkraus , 1992).

The earliest production of fermented foods was based on spontaneous fermentation due to the development of the micro flora naturally present in the raw material. Then, the quality of the end product was dependent on the microbial load and spectrum of the raw material. Spontaneous fermentation was optimized through back slopping, i.e., inoculation of the raw material with a small quantity of a previously performed successful fermentation. Today, the production of fermented foods and beverages through spontaneous fermentation and back slopping represents a cheap and reliable preservation method. The direct addition of selected starter cultures to raw materials has been a breakthrough in the processing of fermented foods, resulting in a high degree of control over the fermentation process and standardization of the end product.

The fermentation of traditional fermented foods is frequently caused by natural, wild-type LAB that originate from the raw material, the process apparatus or the environment and that initiate the fermentation process in the absence of an added commercial starter (Bocker *et al.*, 1995; Weerkamp *et al.*, 1996). Moreover, many traditional products obtain their flavour intensity from the non-starter lactic acid bacteria (NSLAB), which are not part of the normal starter flora but develop in the product, particularly during maturation, as a secondary flora (Beresford *et al.*, 2001). Pure cultures isolated from complex ecosystems of traditionally fermented foods exhibit a diversity of metabolic activities that diverge

strongly from the ones of comparable strains used as industrial bulk starters (Klijn *et al.*, 1995). These include differences in growth rate and competitive growth behaviour in mixed cultures, adaptation to a particular substrate or raw material, antimicrobial properties and flavour, aroma and quality attributes.

Fermentation is of two types, controlled and uncontrolled (traditional) fermentation. In controlled fermentation, beneficial microorganisms were favoured and deleterious organisms are avoided to produce good quality product (Holzapfel *et al.*, 1995). The enzymatic potential of bacteria has been exploited in the production of fermented foods by the inoculation of specific starter cultures. Nowadays, lactic acid starter bacteria are widely used in combination with probiotic (Bifidobacterium, Lactobacillus) bacteria to manufacture fermented dairy products (Vinderola and Reinheimer, 2003). Lactic acid fermentation as a means of food preservation is probably one of the oldest biotechnological processes rooted in the cultural history of mankind (Tamang *et al.*, 2005; Holzapfel *et al.*, 1995) converting biomass, specifically waste materials, into industrial products. . The use of lactic acid fermentation in the preservation of products such as food and feed is well known. Inoculative of suitable LAB ensures rapid acidification and eventual predominance of desired microorganism able to conduct ensilation. During lactic acid bacterial fermentation, it converts the available sugar to acid and thus lowers the pH (Martin, 1996). The low pH inhibits the growth of unwanted microorganisms (Brookes and Buckle, 1992). The acid odour of fermented food products is due to acetic acid and lactic acid produced by it (Bucharles *et al.*, 1984).

Fermentation processes are used in the pharmaceutical industry for the production of amino acids, antibiotics and other fine chemicals (Moueddeb *et al.*, 1996). Montel *et al.* (1998) described the role of lactic acid bacteria and *Micrococcaceae* in flavour development of fermented meat products. Lactic acid fermentation of kitchen waste inhibits the growth of putrefactive bacteria and food poisoning bacteria which as a result enables the preservation and deodorization of the kitchen waste (Wang *et al.*, 2001)

2.2.1. Fermented food products

The main microorganisms used for the production of fermented food products are lactic acid bacteria, yeast and fungi. Lactic acid fermented products are produced primarily on autolytic processes.

LAB have a long and safe history of use as preservatives in dairy fermentations where they are commonly employed as starter cultures, especially in the manufacture of cheese. Flavour development in fermented diary products involves a series of chemical and biochemical conversion of milk components by the main microflora, lactic acid bacteria in these diary products (van Kranenburg *et al.*, 2002). Nisin produced by lactic acid bacteria in cheese products provided protection from contamination with *Listeria monocytogenes*, which poses a serious problem during cheese manufacture and ripening (Sullivan *et al.*, 2002). Dako *et al.* (1995) cited that lactic acid bacteria are major contributors to enzymatic systems in cheese, they also create the conditions of pH and temperature involved in the ripening process.

Commonly found Fermented fish products were fish sauces and pastes. The low salt content in these lactic acid fermented products permits them to consume as a main diet, when compared to high salt fish sauces and pastes. Lactic acid bacteria play an essential role in the production of European type fermented meat products (Hugas and Monfort, 1997). The lactic acid bacteria was used as starter cultures in the preparation of fermented sausages (Callewaert *et al.*, 2000; Foegeding *et al.*, 1992; Hugas *et al.*, 1995; Vogel *et al.*, 1993), fermented vegetables and olives (Harris, 1998; Harris *et al.*, 1992; Ruiz-Barba *et al.*, 1994), and dairy products (Benkerroum *et al.*, 2002; Buyong *et al.*, 1998; Giraffa, 1995; Roberts *et al.* 1992). The common lactic acid bacteria used in the milk industry are *Lactobacillus*, *Lactococcus* and *Streptococcus* (Champomier-Verges *et al.*, 2002)

2.2.2. Starter Cultures

Earliest production of fermented foods depends on the natural micro flora in raw material and so the end products quality varies. The direct addition of selected starter cultures to raw materials has been a breakthrough in the processing of fermented foods, resulting in a high degree of control over the fermentation process and standardization of the end product. The problems of variable quality of the fermentation process can be minimized by the use of starter cultures rather than relying on the natural microbial contaminants (Bacus and Brown, 1985). The use of large number of cells as inoculum or as starter culture helps to discourage the colonization of undesired organism by the mechanism of antagonism (Holzapfel *et al.*, 1995). Starter culture is defined as a microbial preparation of large number of cells of atleast one micro organism to be added to raw material to produce a fermented food by accelerating and stirring its fermentation process (Weinberg and Muck, 1996). So the potential microorganism must be competitive and grow vigorously in the silage, should be homo fermentative and produce maximal amounts of lactic acid in short time, should be acid-tolerant, and be able to grow in material of high dry matter and at temperatures extending to 50°C (Tsigos and Bouriotis, 1995).

2.2.2.1. Selection of starter cultures

Strains with the proper physiological and metabolic features were isolated from natural habitats or from successfully fermented products (Oberman and Libudzisz, 1998). However, some disadvantages have to be considered. In general, the initial selection of commercial starter cultures did not occur in a rational way, but was based on rapid acidification and phage resistance. Thus it can cause preservative effect and affects nutritional value of the product (Weinberg and Muck, 1996). Starter cultures are applied to bring about beneficial metabolic and sensory changes of a food generally accompanied by a preservation effect. Holzapfel *et al.* (1995) tried to compare starter culture and protective culture. For a starter culture, metabolic activity (eg; acid production) has technological importance whilst antimicrobiological action may constitute a secondary effect. But for protective culture, the functional objectives are the

inverse. Originally, industrial starter cultures were maintained by daily propagation. Later, they became available as frozen concentrates and dried or lyophilized preparations, produced on an industrial scale, some of them allowing direct vat inoculation (Sandine, 1996).

Hundreds of selected strains are used as starter cultures in industrial food fermentations. Controlled starter cultures of lactic acid bacteria are of great importance in agro food industry in respect of strain selection, product and process development and characterization of lactic acid bacteria is a critical point. Lactic acid bacteria are traditionally applied as starter cultures for the production of fermented foods. In these products, LAB have two major functions viz; (i) achievement of certain beneficial physicochemical changes in the food ingredients, e.g; acidification, curdling and production of flavour compounds and (ii) inhibition of the outgrowth of microbial pathogens and spoilage organisms (Vereecken and Van Impe, 2002). The species of lactic acid bacteria occupies a central role in these processes as they cause rapid acidification of the raw material through the production of lactic acid. (Caplice and Fitzgerald, 1999; Ray, 1992. Wood 1997; Wood and Holzapfel, 1995). Also their production of acetic acid, ethanol, aromatic compounds, bacteriocins and exopolysaccharides and several enzymes enhance shelf life, improved texture and contribute to the pleasant sensory profile of the end products (Champomier-Verges *et al.*, 2002). *Lactobacillus plantarum* were tried for fermentation because this species meets most of the criteria presented by Whittenbury (1961).

A starter culture of proper lactic acid bacteria has to be added for fermentation because they are present in low number of units biomass in the order 10^1- 10^4/g. (Knachel, 1981). The use of large cell numbers as inoculum enables successful competition of a starter culture during fermentation. Inoculation rates of starter cultures as stated by the manufacturers are usually 10^5-10^6 viable cells/g, which is often sufficient for the inoculant LAB to outgrow the epiphytic LAB and become the predominant population in the silage. Pahlow (1991) has suggested an inoculation factor (IF) of 2 (two-fold increase in LAB) in order to achieve a consistent positive effect.

Deproteinisation and demineralization of shrimp shells for the production of chitin was done using different strains of *Aspergillus niger* by Teng *et al.* (2001). He screened 34 fungal strains of deuteromycetes and zygomycetes based on their protease activity. This was done in corn meal agar with gelatin as the substrate and clearing in the agar medium indicate degradation. Knachel (1981) tried three lactic starter *Pediococcus pentosaccus*, *Staphylococcus caenosus* and another species identified as Lactobacillus isolated from tropical water shrimp by Dr. Zainoha Zakaria, University of Loughborough for fermentation of shrimp waste for astaxanthin extraction. The application of bacteriocinogenic lactic strains as starter cultures in fermented products could provide an additional tool for preventing the outgrowth of food pathogens in sausages as well as enhancing the competitiveness of the starter organism (Hugas and Monfort, 1997).

Weinberg and Muck (1996) suggested that mixed strain inoculants of *Lactobacillus plantarum* with *Lactobacillus acidophilus*, *Pedicoccus acidilactici* and *Pedicoccus pentosaceus* results better than with individual inoculum. Fitzsimons *et al.*, (1992) screened Pediococcus strain for potential use as inoculants in grass ensilage. One strain (*Pediococcus acidilactici* G 24) was most effective and stimulated the epiphytic *Lactobacillus plantarum* population in ensiled grass with high carbohydrate content. Grant *et al.* (1994) isolated a strain of *Lactobacillus plantarum* from pickle fermentation, which was found efficient in producing grass legume silage. Mixed bacterial inoculants containing *Entreococcus faecum* and *Lactobacillus plantarum* were used for fermenting shrimp waste with dry molasses by Evers and Caroll (1996).

Fenlon *et al.* (1993) proved that *Pediococcus acidilactici*, which is used as starter culture in grass silages, inhibits *Listeria monocytogenens* in the initial 14 days of fermentation. Protease producing Bacillus can be used for deproteinisation of crustacean waste (Yang *et al.*, 2000). Wang and Chio (1998) used *Pseudomonos aeruginosa* K-187 for the deprotenisation of shrimp and crab shell. Shimahara and Takiguchi (1988) studied the efficiency of *Pseudomonos maltophilia* for deproteinization of demineralized shell chips from various sources. Teniola and Odunfa (2001) used *Saccharomyces cerevisiae* and a

heterofermentative *Lactobacillus brevis* as starter cultures in their fermentation study of *Ogi*.

A lactobacillus spp strain B_2 isolated from shell fish waste at an inoculum level of 5% v/w was used by Cira *et al.* (2002) for fermentation of shrimp bio waste for chitin recovery. He selected this strain based on their acidification rate and fermentative nature. Shirai *et al.* (2001) optimized the inoculum levels of lactic acid bacteria for shrimp ensilation. He found that acid production was not significantly different when 5 and 10% inoculum levels were applied. Thus the cost of the process can be reduced using less quantity of inoculum. Albrecht *et al.* (1992) used combination of strains of lactic acid bacteria species isolated from grass silages. Among the species tried, are *Lactobacillus.delbrueckii*, *Lactobacillus casei*, *Lactobacillus rhamnosus* (Takeda and Katsura, 1964). In addition to standard inoculant species such as Lactobacillus *plantarum* and Pediococcus *acidilactici*. Fagbenro and Bello-Olusoji (1997) used *Lactobacillus plantarum* for fermentation of silver prawn, *Macrobrachium vollenlovenii*, waste for silage preparation. Heterofermentative *Lactobacillus buchneri* was used as starter culture in corn silage (Weinberg and Muck, 1996).

Hall and de Silva (1994) reported lactic acid bacteria in their fermentation studies for the preparation of chitin. Bustos and Healy (1994) obtained chitin by the deproteinization of shrimp shell waste with various proteolytic microorganisms including *Pseudomonas maltophilia*, *Bacillus subtilis*, *Streptococcus faecium*, *Pediococcus pentosaseus* and *Aspergillus oryzae*. Rattanakit *et al.* (2003) reported the utilization of shrimp shell waste as a substrate of solid state cultivation of Aspergillus sp. *Lactobacillus paracasei* strain A3 isolated from shell fish waste was used for fermentation of lobster waste as reported by Zakaria *et al.* (1998).

Lactobacillus plantarum has been used as a starter culture for fermentation of vegetables (Fleming *et al.*, 1985; Mac Kay and Baldwin, 1990) and sausage products (Bacus and Brown, 1985; Hugas *et al.*, 1993). Bacterial components of starters consist of Staphylococci and lactic acid bacteria (*Lactobacillus* and *Pediococcus*) and are well known for the acidification of the

sausages (Hammes *et al.*, 1990). Blanco (1986) used *Lactobacillus pentosus* for batch-fed fermentation process for the production of lactic acid from whey lactose. Combinations of different bacterial strains belonging to the genera Lactobacillus, Streptococcus and Bifidobacterium, have been used traditionally in the preparation of fermented dairy products (Prasad *et al.*, 1998; Dunne *et al.*, 1999). *Lactobacillus plantarum* and other lactic acid bacteria (LAB) have been reported as the prevalent microorganisms associated with the spontaneous fermentation of cassava starch (Figueroa *et al.*, 1995 & 1997; ben Omar *et al.*, 2000; Ampe *et al.*, 2001). The α-amylase producing strain of *Lactobacillus plantarum* was used by Pintado *et al.* (1999) for the utilization mussel processing waste. From the studies of Idler *et al.* (1994), it was found that *Lactobacillus casei* produced the highest levels of lactic acid and lowest pH than *Lactobacillus rhamnosus*. Combination of starter cultures of *Lactobacillus casei* and *Lactobacillus rhamnosus* improved the digestibilities of crude protein and crude fiber.

2.2.3. Lactic acid Bacteria

The lactic acid bacteria (LAB) emerged around 3 billion years ago, probably before the photosynthetic cyanobacteria. Their expansion has really begun with the apparition of milk producing mammals, over 65 million years ago. However, the first registered usage came from the discovery of small vases punched by small holes, near the Neufchatel Lake, over BC 3000. Since these days, humans were able to control milk curdling (Champomier-Verges *et al.*, 2002). The genus *Lactobacillus* presently comprises more than 50 recognized species of non pathogenic bacteria which are useful to human in several respects: they are indispensable agents for the fermentation of foods and feed, and they exert probiotic effects in human and animals.

Lactic acid bacteria (LAB) comprise a diverse group of Gram-positive, nonsporeforming bacteria (Kandler and Weiss, 1986). They generally lack catalase; although in rare cases pseudocatalase can be found. They occur as cocci and rods and are chemoorganotrophic and grow only in complex media.

LAB, which include the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium* and *Propionibacterium* (Sullivan *et al.*, 2002), play an essential role in food fermentations given that a wide variety of strains were routinely employed as starter cultures in the manufacture of dairy, meat and vegetable products. The genera *Lactobacillus, Leuconostoc* and *Pediococcus* were traditionally treated separately because of their different morphology. However, phylogenetically they are intermixed. There is no good correlation between the phylogenetic relatedness within the genus *Lactobacillus* and their biochemical and physiological based subdivisions into the subgenera *Thermobacterium, Streptobacterium* and *Betabacterium* or homofermentatives and heterofermentatives (Collins *et al.*, 1991; Hammes *et al.*, 1991; Schleifer and Ludwig, 1994). After the morphological characteristics were known, the entire group of LAB was split into 3 groups that were treated separately for identification. Lactobacilli are rod shaped bacteria, and the group consisting of *Streptococcus*, *Lactococcus*, *Enterococcus* and *Leuconostoc* from *Cocci* occur as chains of pairs (Wijkzes *et al.*, 1997).

Lactic acid bacteria is generally recognized as food grade organism and involved in the production of various fermented milk products, vegetables and meat products and the processing of other products like wine. It is generally recognized as safe organism and there is no indication of a health risk of LAB involved in food fermentations (Holzapfel *et al.*, 1995). Lactic acid bacteria and specifically *Lactobacilli* are good candidates as probiotic strains because they are normal components of the gut microflora (Hugas and Monfort, 1997) There is some debate as to whether the concept of probiotic should include dead microorganisms, or even bacterial fragments (Vinderola and Reinheimer, 2003). Naidu *et al.* (1999) introduced the concept of 'Probiotic- Active Substance', as a cellular complex of lactic acid bacteria that has a capacity to interact with the host mucosa and may beneficially modulate the immune system independent of viability of lactic acid bacteria. They have antagonistic properties towards pathogenic bacteria either by antimicrobial substance production or competitive exclusion. Because of its probiotic nature, daily intake of lactic acid bacteria in the diet is very necessary. So it is used in the development of wide range of

fermented foods (Hugas and Monfort, 1997). Reports on the involvement of LAB in human infections (Aguirre and Collins, 1993) indicate that some species may act as opportunistic pathogens in rare cases.

A generic computerised system for the identification of bacteria was developed by Wijtzes *et al.* (1997). The system was equipped with a key to the identification of lactic acid bacteria. The identification was carried out in two steps. The first step distinguished groups of bacteria by following a decision tree with general identification tests. The second step in the identification was the distinction of species within a group on the basis of biochemical fermentation patterns. Another identification system was described by Cox and Thomsen (1990) assess the entire group of lactic acid bacteria as a whole.

Fermentable carbohydrates were used as energy sources by LAB and were degraded to lactate (homofermentatives) or to lactate and additional products such as acetate, ethanol, carbon dioxide, formate or succinate (heterofermentatives). They play an essential role in food technology. They could improve the aroma and texture of food and inhibit the growth of spoilage bacteria. However, not all of the LAB are useful, some of them are involved in food spoilage or may even be pathogens (Schleifer *et al.*, 1995). Lactic acid bacteria act as natural food preservatives to improve food safety and stability. The reduction of pH and the removal of large amounts of carbohydrate by fermentation provide the major preservative effect in fermented foods (Daeschel, 1989).

Knowledge of the genetics and molecular biology of lactic acid bacteria had been advanced rapidly during the last decade. Genetically modified M-LAB for food fermentation have been constructed with improved technical properties such as proteolytic activity, aroma production and carbohydrate fermentation (Lindgren, 1999). Fourier transform mid-infrared (FT-MIR) spectroscopy was used to determine the concentrations of substrate, major metabolites and lactic acid bacteria in fermentation processes (Fayolle *et al.*, 1997)

2.2.3.1. Proteolytic System of Lactic Acid Bacteria

The lactic acid bacteria have a complex proteolytic system capable of converting protein to the free amino acids and peptides necessary for growth and acid production. The proteolytic system is composed of a proteinase, which is involved in the initial cleavage of protein; peptidases, which hydrolyse the large peptides thus formed and transport systems which are involved in the uptake of small peptides and amino acids (Law and Haandrikman, 1997). The fraction of nitrogen present as free amino acids increases and the fraction present as polypeptides decrease as a result of the hydrolysis process.

The proteinase of lactococci has a cell-envelope location (Law and Kolstad, 1983) and requires Ca^{2+} for stable attachment to the cell envelope. So, proteinase activity can be released by the removal of calcium (Mills and Thomas, 1978). It was shown that peptidases are in most cases intracellular enzymes (Kamaly and Marth, 1989; Khalid and Marth, 1991; Pritchard and Coolbear, 1993; Visser, 1993), which indicate the importance of cellular lysis. Bie and Sjostrom (1975) reported that sodium ions promote the autolytic properties of lactic acid bacteria used in cheese making while calcium and magnesium retard the process. Dako *et al.* (1995) in their study demonstrated that Lactobacillus seemed to autolyse more rapidly than the other lactic acid bacteria tested and to liberate their intracellular enzymes and proteins in the external environment. According to Zakaria *et al.* (1998), the process of protein solubilisation is due to the action of proteases released from the gastrointestinal tract of the lobster and also of cathepsins from muscle tissue, both of which are active at acidic pHs.

2.2.3.2. *Lactobacillus plantarum*

Lactobacillus plantarum (Fig. 2.4) is a widespread member of the genus *Lactobacillus*, commonly found in sauerkraut, pickles, brined olives, Korean kimchi, Nigerian ogi, sourdough and other fermented plant materialsand also in some cheeses and fermented sausages. It is also present in saliva (from which it was first isolated). This microorganism is Gram (+), grows at 15 but not at 45 °C, and produces both isomers of lactic acid (D and L). It has the ability to liquefy

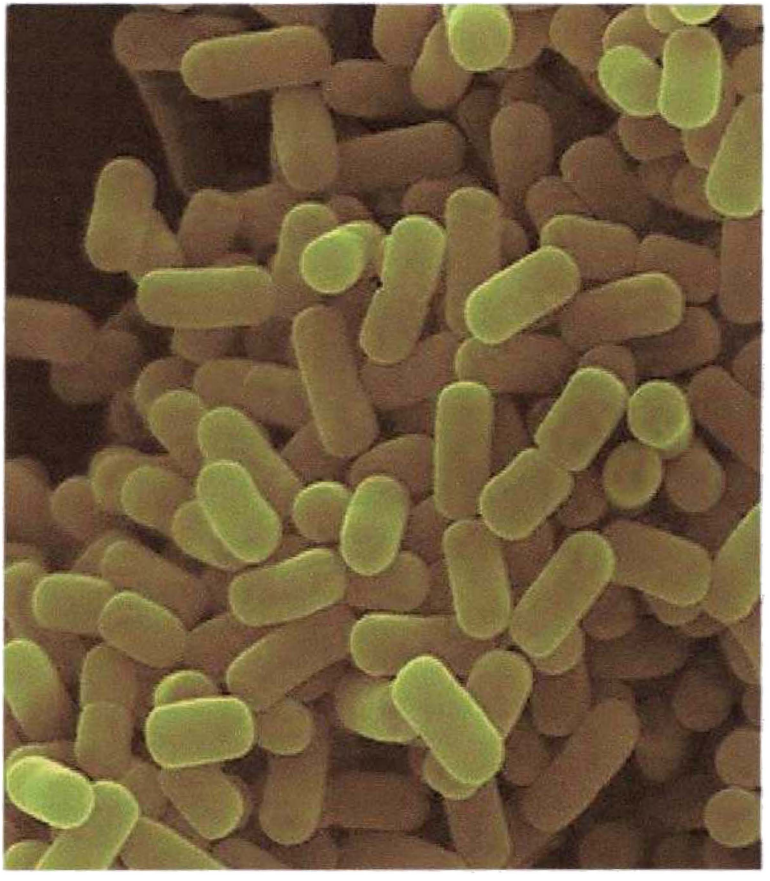


Fig. 2.4. *Lactobacillus plantarum*

gelatin. *L. plantarum* has one of the largest genomes known among the lactic acid bacteria and is a very flexible and versatile species.

L. plantarum and related lactobacilli are unusual in that they can respire oxygen but have no respiratory chain or cytochromes—the consumed oxygen ultimately ends up as hydrogen peroxide. The peroxide probably acts as a weapon to exclude competing bacteria from the food source. In place of the protective enzyme superoxide dismutase present in almost all other oxygen-tolerant cells, this organism accumulates millimolar quantities of manganese polyphosphate. Because the chemistry by which manganese complexes protect the cells from oxygen damage is subverted by iron, these cells contain virtually no iron atoms; in contrast, a cell of *Escherichia coli* of comparable volume contains over one million iron atoms. *L. plantarum* is the most common bacterium used in silage inoculants.

2.2.3.3. *Lactobacillus brevis*

Lactobacillus brevis (Fig. 2.5) is a heterofermentative bacterium that utilizes hexoses by the 6-phosphogluconate pathway, producing lactic acid, CO_2 and ethanol and/or acetic acid in equimolar amounts (Kandler, 1983). It can be isolated from many different environments and it is frequently used as starter culture in silage fermentation, sourdough and lactic-acid-fermented types of beer. In beverages obtained by alcoholic fermentation, lactobacilli may contribute to the quality of the product but may also cause spoilage. Certain *L. brevis* strains are resistant to hop bittering substances such as isohumulone and are able to grow in beer. Their growth changes the turbidity, flavor and aroma of the beer (Richards and Macrae, 1964). *L. brevis* strains involved in wine fermentation may produce biogenic amines by decarboxylation of the precursor amino acids through the action of substrate-specific enzymes. The ingestion of foods containing high levels of such amines, particularly histamine and tyramine, can lead to several toxicological disturbances (ten Brink *et al.*, 1990; Marine-Font *et al.*, 1995).

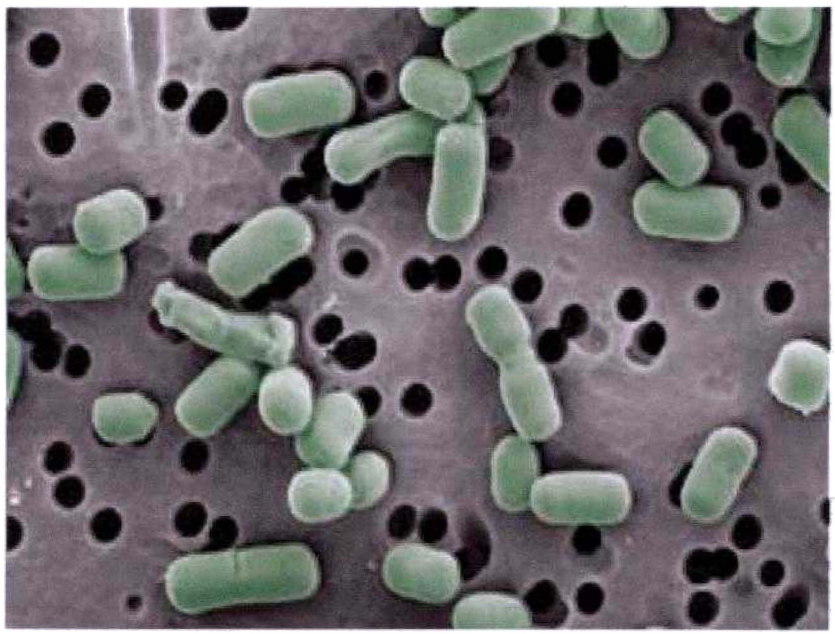


Fig. 2.5. *Lactobacillus brevis*

2.2.3.4. Metabolic Products of Lactic Acid Fermentation

Lactic acid bacteria is recognized as industrially important organisms as they are involved in the preservation of food and production of many fermented food substances (ten Brink *et al.*, 1994). Various metabolic products produced by lactic acid bacteria are responsible for its preservative nature. Since food safety has become an increasingly important international concern, the application of anti microbial peptides from lactic acid bacteria that target food pathogens without toxic or other adverse effects has received great attention (Takeda and Abe, 1962).

Lactic acid, the main product of lactic fermentation, predominates over other antimicrobials produced by lactic acid bacteria. Various metabolic products like organic acids, lactic acid and acetic acid, hydrogen peroxide, enzymes like lactoperoxidase system with H_2O_2, lysozyme low-molecular metabolites like reuterin (3-hydroxy propionaldehyde), diacetyl and fatty acids and bacteriocins like nisin and others are produced by lactic acid bacteria have antimicrobial properties (Holzapfel *et al.*, 1995).

Lactic acid is the most common fermentation product, which reduces the pH and inhibits many putrefactive bacteria and toxigenic bacteria. The produced acid solubilises the fat and diffuses to the bacterial cell, reduces the intracellular pH and thus slows down the metabolic activity of bacteria (Brown and Booth, 1991). Because of the higher dissociation constant of acetic acid (pKa, 4.75), it shows greater inhibition of putrefactive and toxigenic bacteria than lactic acid produced (pKa-3.1) (Holzapfel *et al.*, 1995).

During lactic acid fermentation, it took more than 5 hours for the bacteria to convert glucose to lactic acid and this delay leads to spoilage. So an initial pH adjustment was required till the production of sufficient acid to lower the pH. Rao *et al.* (2000) used acetic acid, citric acid, lactic acid and hydrochloric acid for initial pH adjustment and to prevent spoilage in their fermentation study.

Hugas and Monfort (1997) found that the strains of *Lactobacillus curvatus,. Lactobacillus sake, Lactobacillus bavaricus* and *Lactobacillus plantarum* produce bacteriocins, which are antimicrobial compounds of a peptidic nature, active against different indicator bacteria. Bacteriocins are proteinaceous compounds produced by bacteria that exhibit a bactericidal or bacteriostatic mode of action against sensitive bacterial species (Klaenhammer, 1988; Nettles and Barefoot, 1993). Bacteriocins of *Lactobacillus plantarum* have a major limitation because of their narrow inhibitory spectrum which does not include various food borne pathogens (EnarPb *et al.*, 1996).

Huttunen *et al.* (1995) reported that the production of a certain non-protein amino acid (PCA) is also involved in the anti microbial action of lactic acid bacteria. Among lactic acid bacteria, only *Streptococcus bovis* had been reported to produce PCA by conversion of glutamine (Chen and Russell, 1989).

According to Holzapfel *et al.* (1995), heterofermentative LAB like *Leuconostoc* spp. and some lactobacilli produce acetic acid also along with lactic acid from hexoses. Kandler (1983) reported that under specific conditions of hexose limitation and or availability of oxygen, homofermentative LAB (eg; *Pediococci, Lactococci* and most *Lactobacillus* spp.) may dissimilate lactic acid to acetic acid, formic acid and or CO_2. Some of the other products are H_2O_2, enzymes, low molecular weight metabolites like diacetyl, 3-hydroxy propenaldehyde etc. Fayolle *et al.* (1997) used Fourier transform mid infra red spectroscopy to determine the concentrations of substrate, various metabolites, lactic acid bacterial concentrations etc.

2.2.3.5. Carbohydrates as Substrates for Lactic Fermentation

Microbial processes generally require carbohydrates as sources of energy, therefore the lactic fermentation would be retarded when food wastes of animal origin was only used as substrate. Free sugar is an essential substrate for the growth of lactic acid bacteria (Raa *et al.*, 1983). The main source of sugar for lactic acid bacteria in milk is lactose (Adamberg *et al.*, 2003).

Many studies have been presented regarding the use of substrates as sources of energy for lactic acid fermentation (Martin, 1996 a, b). Martin and Bemister (1994) used peat extract as carbohydrate source for lactic acid fermentation in the production of fish. Molasses have been used as sugar source for lactic acid fermentation of shrimp waste and crab waste for the preparation of silage by Abazinge *et al.* (1986). Evers and Carroll (1996) in their study, molasses was chosen because it assisted in the fermentation process, was relatively inexpensive, had a high potential consumer acceptance by the seafood and livestock industry, and was acceptable to animals. Hemicelllose rich wheat was partially hydrolyzed to pentose sugar during ensilage preparation using *Lactobacillus pentosus*.It is capable of converting these pentose sugar formed to acetic acid and lactic acid during fermentation (Weinberg and Muck, 1996). Fagbenro and Bello-Olusoji (1997) used molasses and cassava starch as carbohydrate source for the fermentation of shrimp head using *Lactobacillus plantarum*. Dominguez (1988) and Green *et al.* (1983) suggested 20% and 30% molasses respectively for lactic acid fermentation. But if roots are using as principal sources of carbohydrates, then the ratio will differ. Roots 50-30%, molasses 10% and fish wastes 40-60% was suggested by Dominguez (1988).

In some traditional lactic fermented products, cooked rice, sacharified rice, or cassava flour were added as source of sugar, to promote a satisfactory fermentation. LAB generally thought to be amylase negative and so glucose or sucrose considered more suitable utilizable substrate than potato starch. Minced lobster waste was thoroughly mixed with 10% w/w glucose and 10% v/w of the prepared inoculum of LAB for fermentation (Zakaria *et al.*, 1998). Bautista *et al.* (2001) used 10% w/w glucose for lactic fermentation of shrimp waste for astaxanthin extraction. Fresh crab waste has been successfully ensiled with molasses and straw and fed to sheep (Samuels *et al.*, 1991; Abazinge *et al.*, 1994). Martin and Bemister (1994) found that peat extract was appropriate carbohydrate source for LAB. Glucose was used as source of carbohydrate by many authors in their study (Zakaria *et al.*, 1998; Rao *et al.*, 2000; Shirai *et al.*, 2001). Cira *et al.* (2002) tried sucrose, lactose or whey powder as carbohydrate source in his studies of Lactobacillus fermentation of shrimp shell waste. From his studies, he fixed the minimum carbohydrate concentration at 10% wet weight

basis. He found that sucrose and lactose were good sources of carbohydrate for fermentation as they promoted high acidification. A pH drop from an initial pH 7.5 to 4.4-4.8 was achieved after 96 hr fermentation in both the cases. But whey powder, which also contained lactose was not that much efficient in the production of acid. Yang *et al.* (2000), in his experiments with *Bacillus subtilis* for the production of protease, different carbon sources like glucose, lactose, carboxymethyl cellulose, arabinose, xylose, cellulose, rice bran etc. were tried. It was found that production of protease was greatly enhanced by the addition of lactose or arabinose. And 1% arabinose was the most effective substrate and was added to the media for the study.

2.2.4. Bacillus

Proteases produced by Bacillus species including *Bacillus* spp, *Bacillus subtilis, Bacillus firmus* are by far the most important group of enzyme being exploited for degradation of proteinaeous waste into useful biomass (Atalo and Gashe, 1993, Venugopal *et al.* 1989). The enzyme protease in bacillus was most active at pH-8 and 50^0C. Most of the protease used in waste bioconversion are alkaline proteases (Atalo and Gashe 1993; Venugopal *et al.* 1989).

According to Lauer *et al.* (2000) commercial bacterial proteases were derived from Bacillus fermentation broth. Bacillus species generally synthesise a variety of extracellular enzymes (e.g., proteinases and lipases), the maximum synthesis of which normally occurs in the late exponential and early stationary phases of growth, before sporulation (Priest, 1977).

Vachova and Kucerova (1998) studied the effect of calcium as regulator of *Bacillus megaterium* cytoplasmic proteolytic activity. *Bacillus cereus* is also involved in the fermentation of cassava starch. The presence of *Bacillus cereus* in fermented cassava starch is important as this species produces toxins and may be causing food poisoning when consumed in numbers greater than 5.0 log CFU g^{-1}. Payot *et al.* (1999) standardized the conditions of fermentation with *Bacillus coagulans* for the production of lactic acid.

2.2.4.1. *Bacillus subtilis*

Bacillus subtilis (Fig. 2.6) is a Gram-positive, catalase-positive bacterium commonly found in soil. A member of the genus *Bacillus*, *B. subtilis* has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions. Unlike several other well-known species, *B. subtilis* has historically been classified as an obligate aerobe, though recent research has demonstrated that this is not strictly correct (Nakano and Zuber, 1998).

It has also been called *Bacillus globigii*, Hay bacillus or Grass bacillus. It is bacillus because the bacterium is rod shaped or bacilli shaped. *B. subtilis* is not considered a human pathogen; it may contaminate food but rarely causes food poisoning. *B. subtilis* produces the proteolytic enzyme subtilisin. *B. subtilis* spores can survive the extreme heating that is often used to cook food, and it is responsible for causing *ropiness* — a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides — in spoiled bread dough.

B. subtilis can divide asymmetrically, producing an endospore that is resistant to environmental factors such as heat, acid, and salt, and which can persist in the environment for long periods of time. The endospore is formed at times of nutritional stress, allowing the organism to persist in the environment until conditions become favorable. Prior to the decision to produce the spore the bacterium might become motile, through the production of flagella, and also take up DNA from the environment.

Bacillus subtilis is a psychrotophic micro organism (Kohlmann *et al.*, 1991) and secrete a thermo resistant protease (Poffe and Mertens, 1988), which may cause proteolysis in pasteurized or sterilized milk. *Bacillus subtilis* is an important starter culture for Asia and African fermented soyabean like Japanese natto, Thai Thua-nao, Indian kinema and West African dawadawa (Ohta and Natto, 1986; Steinkraus, 1995; Sarkar *et al.*, 1993). But the protease produced by *Bacillus subtilis* is a neutral protease as indicated by its pH activity and stability at neutral range. These fermentation processes were characterized by

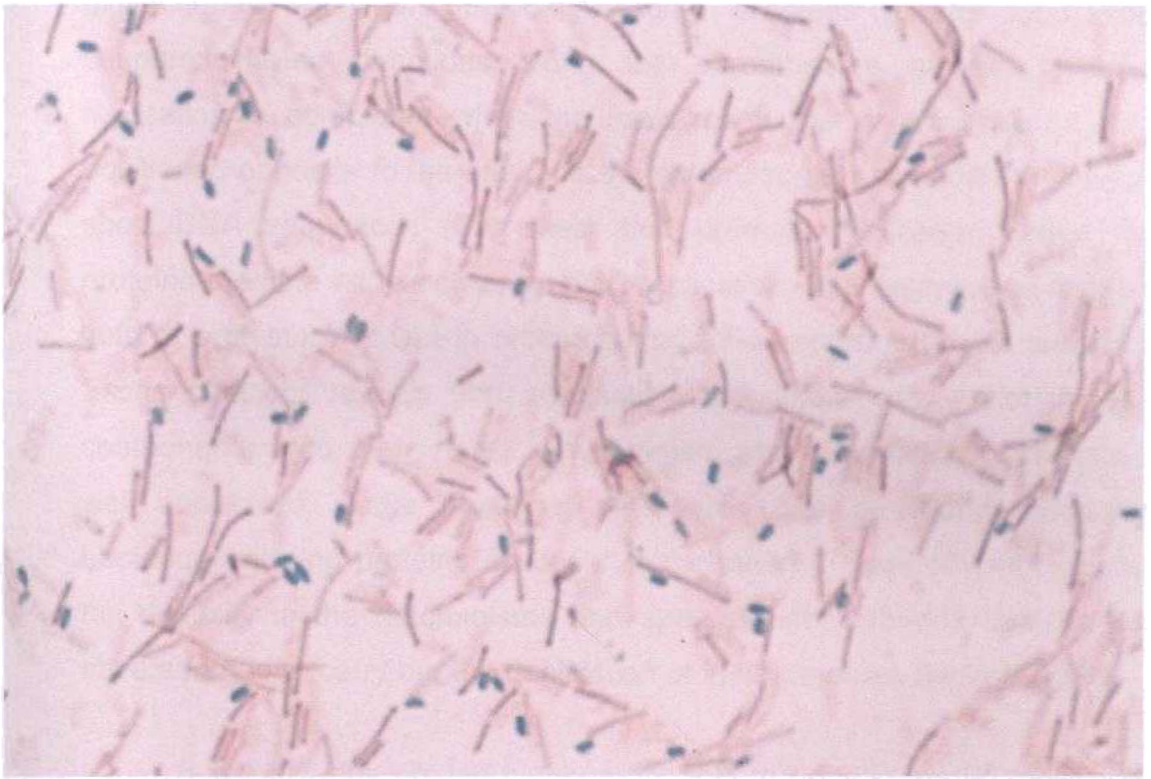


Fig. 2.6. Spore of *Bacillus subtilis*

conversion of legume proteins to amino acids and its utilisation led to the production of ammonia and a rise of pH. Bacillus fermentation of legumes was reported by many authors (Odunfa and Dawadawa, 1986, Sarkar and Tamang, 1995). Enzymes prepared from *Bacillus subtilis* were used for the proteolysis of whey proteins (Castro *et al.*, 1996).

Bacillus subtilis duplicates its single circular chromosome by initiating DNA replication at a single locus, the origin (*oriC*). Replication proceeds bidirectionally and two replication forks progress in the clockwise and counterclockwise directions along the chromosome halves. Chromosome replication is completed when the forks reach the terminus region, which is positioned opposite to the origin on the chromosome map, and contains several short DNA sequences (*Ter* sites) that promote replication arrest. Specific proteins mediate all the steps in DNA replication. The comparison between the sets of proteins involved in chromosomal DNA replication in *B. subtilis* and in *Escherichia coli* reveals both similarities and differences. Although the basic components promoting initiation, elongation, and termination of replication are well conserved, some important differences can be found (such as one bacterium missing proteins essential in the other). These differences underline the diversity in the mechanisms and strategies that various bacterial species have adopted to carry out the duplication of their genomes (Noirot, 2007).

B. subtilis has proven highly amenable to genetic manipulation, and has therefore become widely adopted as a model organism for laboratory studies, especially of sporulation, which is a simplified example of cellular differentiation. It is also heavily flagellated, which gives *B.subtilis* the ability to move quite quickly. In terms of popularity as a laboratory model organism *B. subtilis* is often used as the Gram-positive equivalent of *Escherichia coli*, an extensively studied Gram-negative rod.

B. subtilis is used as a soil inoculant in horticulture and agriculture. *B. subtilis* has been used for a biowarfare simulant during Project SHAD (aka *Project 112*) (Madigan and Martinko, 2005). *B. subtilis* hazard status is under dispute (Nakano and Zuber, 1998). Enzymes produced by *B. subtilis* and *B.*

licheniformis are widely used as additives in laundry detergents.It is used as a model organism for laboratory studies. A strain of *B. subtilis* formerly known as *Bacillus natto* is used in the commercial production of the Japanese delicacy natto as well as the similar Korean food cheonggukjang. *B. subtilis* strain QST 713 (marketed as QST 713 or Serenade™) has a natural fungicidal activity, and is employed as a biological control agent. It can convert explosives into harmless compounds of nitrogen, carbon dioxide, and water. It plays a role in safe radionuclide waste [e.g. Thorium (IV) and Plutonium (IV)] disposal with the proton binding properties of its surfaces. Recombinants *B. subtilis* str. pBE2C1 and *B. subtilis* str. pBE2C1AB were used in production of polyhydroxyalkanoates (PHA) and that they could use malt waste as carbon source for lower cost of PHA production.

Kiers *et al.* (2000) reported the effect of fermentation of soyabean using pure cultures of Bacillus sp on the solubilisation and degradation of soybean that may result in increased invitro digestibility. It resulted in more or less complete breakdown of storage proteins in soybean, which showed the proteolytic activity of Bacillus sp (Sarkar *et al.*, 1993; Sarkar and Tamang, 1995). Maximum proteolytic activity was reached after 12-24 hours and it remained fairly constant during prolonged fermentation (Allagheny *et al.*, 1996; Sarkar *et al.*, 1993). Degradation of carbohydrate to low molecular weight compounds also occurred due to fermentation. The major metabolic activity of *Bacillus subtilis* was the proteolysis of proteins and utilization of released aminoacids and thus ammonia was produced. (Allagheny *et al.*, 1996). This was responsible for the pH rise and for the unpleasant strong ammonia odour and spoilage of the fermented product was occurred (Campbell-Platt, 1980; Odunfa and Dawadawa, 1986; Sarkar *et al.*, 1993). The production of ammonia is a consequence of the utilisation of amino acids by the bacteria as sources of carbon and energy:

$RCH(NH_3^+)COO^- + nO_2 \rightarrow nCO_2 + NH_2O + NH_4^- + OH^-$ (Allagheny *et al.*, 1996).

The ammonium production has to be controlled by restricting the growth of bacteria and thus off odour can be regulated.

Kim *et al.* (2002) studied and characterized the proteases produced by *Bacillus stearothermophilus* and the enhancement of their activity by divalent cations of calcium and iron. Serine proteases are excreted by this Bacillus species. An optimum temperature of 75^{0}C and pH of 7.5 were noticed for its maximum activity.

Chen *et al.* (2004) isolated seven Bacillus strains from milk production lines and found that these proteinase and lipase were likely to survive any heat treatments. So, these enzymes remain active in milk powder during storage. Different types of proteinases like extracellular serine proteinases and metallo proteinases were produced by the Bacillus species (Egorov *et al.*, 1982; Chopra and Mathur, 1985). The synthesis of extracellular serine proteinase had been associated with Bacillus sporulation (Egorov *et al.*, 1982; Strongin and Stepanov, 1982).

Bacillus species have been successfully used in the degradation of proteinaceous waste into useful biomass (Atalo and Gashe, 1993). The application of *Bacillus* spp. for the deproteinisation of marine crustacean wastes rarely seen. Yang *et al.* (2000) tried *Bacillus subtilis* and *Pseudomonas maltophilia* for the deproteinisation of crustacean wastes like shrimp shell, crab shell and lobster shell for the production of chitin. Results showed that *Bacillus subtilis* were more efficient than *Pseudomonos maltophila*. Bustos and Healy (1994) demonstrated that chitin obtained by the deproteinization of shrimp shell waste with various proteolytic microorganisms including *Pseudomonas maltophilia*, *Bacillus subtilis*, *Streptococcus faecium*, *Pediococcus pentosaseus* and *Aspergillus oryzae*, had higher molecular weights compared to chemically prepared shellfish chitin.

2.2.5. Degree of Deprotenisation and Demineralisation of Fermented Residue

Chitin in the exoskeleton of shrimp shells is associated intimately with proteins; therefore, deproteinization in the chitin extraction process is crucial. The use of microorganisms or proteolytic enzymes for deproteinization of marine

crustacean wastes is a current trend in conversion of wastes into useful biomass. It is a simple and inexpensive alternative to chemical methods employed in the preparation of chitin (Yang *et al.*, 2000). During fermentation, protein and calcium removal is achieved by enzymatic action on the tissues and by solubilisation of calcium by organic acids, respectively (Healy *et al.*, 1994; Shirai *et al.*, 1997).

About 55% of protein was removed from shrimp shell on fermentation with *Pseudomonos aeruginosa* K-187 for 5 days (Wang and Chio, 1998). According to Shimahara and Takiguchi (1988), treatment of shrimp shell with *Pseuodomonas maltoplila* removed about 60% protein and from crab shell about 80% protein when fermented for eight days. An earlier study (Yang *et al.*, 2000) reported that about 88% of proteins were removed from shrimp shell on fermentation with *Bacillus subtilis* and 73% with *Pseudomonos maltoplila*. But in acid treated shrimp shell, protein removal was only 76% with *Bacillus subtilis* and 71% with *Pseudomonos maltoplila*. Bustos and Michael (1994) have compared the effects of microbial and enzymatic deproteinization and found that a maximum value of 82% deproteinization was achieved with *Pseudomonos maltophilia* after 6 days of incubation.

Hall and de Silva (1994) stated that the fermentation of crustacean shells with lactic acid bacteria lowered the pH of the medium to approximately pH 4, facilitating the hydrolysis of proteins while leaving the associated chitin intact. About 82% of proteins were removed from cray fish waste when fermented with *Lactobacillus pentosus* for the production of chitin. The proteolytic enzymes in fungal strain released aminoacids and act as nitrogen source and anticipated to deprotenise and demineralise the shrimp shell (Bautista *et al.*, 2001; Teng *et al.*, 2001). The residual protein in shrimp shell after fermentation was only about 5%. About 72% of protein is removed form shrimp shell on fermentation with lactobacillus parcasei according to Zakaria *et al.* (1998). Cira *et al.* (2002) reported 87.6% of deprotenisation from shrimp shell in his fermentation study using *Lactobacillus* spp.

2.2.6. Demineralisation

About 61% of calcium was solubilised from lobster shell on fermentation with *Lactobacillus paracasei* according to the work of Zakaria *et al.* (1998). Bautista *et al.*, (2001) reported that approximately 90% of the mineral (carbonates and phosphates) present at the start of fermentation in the crayfish chitinous fraction was solubilized. In his study demineralization proceeded at a relatively steady rate until 36 hour and then continued more slowly until the end of fermentation. About 85% demineralization attained in shrimp shell fermented with lactic acid bacteria (Cira *et al.*, 2002).

2.2.7. Changes in Glucose Concentration During Fermentation

About 88.7% of glucose has been utilized for fermentation as reported by Zakaria *et al.* (1998) when *Lactobacillus paracasei* was used. This value together with pH decline showed that glucose was converted to acidic end products. Studies revealed that *Lactobacillus paracasei* was homofermentative with lactic acid as major end product. Rao *et al.* (2000) reported an optimum glucose concentration of 5% w/v required for lactic acid bacterial fermentation of shrimp bio waste. Addition of glucose to fermenting lactobacillus led to a pH reduction after 4-6 hours. According to Shirai *et al.* (2001), initial glucose concentration was a critical factor of lactic acid fermentation and at concentration lower than 7.5% of glucose, pH did not reach values lower than 5.6. Cira *et al.* (2002) carried out pilot scale lactic acid fermentation of shrimp waste for chitin recovery with minimum 10% w/w glucose concentration. According to them, the total soluble sugars decreased from concentration 80 mg/g to 15 mg/g after 6 days of fermentation as a consequence of conversion to lactic acid and loss due to solubilisation in the liquor.

2.3. Biogenic Amines

Biogenic amines are a group of biologically active organic compounds normally produced by decarboxylation of free amino acids (den Brinker *et al.*, 1996). The presence of biogenic amines in these foods is an indication of food

spoilage, which is dependent upon the availability of free amino acids, the presence of decarboxylase positive microorganisms (bacteria containing enzymes which can decarboxylate free amino acids) and conditions favouring bacterial growth (Halasz *et al.*, 1994). Beddows (1985) cited that during the fermentation process, the content of free amino acids increased. This indicates that the fermentation process has the potential to produce biogenic amines. In order for biogenic amines to be produced, free amino acids and bacteria containing decarboxylase enzymes need to be present together with suitable temperatures and pH. Biogenic amines and their precursors in decarboxylation reactions (Paterson *et al.*, 1990) were given in Table 2.1.

Table 2.1. Biogenic amines and their precursors in decarboxylation reactions.

AMINO ACID	BIOGENIC AMINE
Histidine	Histamine
Ornithine	Putrescine → Spermidine
Lysine	Cadaverine
Arginine	Agmatine → Putrescine

According to Australian Food Standards, the limit of histamine to be present in fish sauces and other fermented products is 100mg/kg. The intestinal tracts of humans contain the enzymes diamine oxidase (DAO) and histamine-N-methyl transferase (HMT) which convert histamine to harmless degradation products. For large doses of histamine, the capacity of DAO and HMT to detoxify histamine is limited, resulting in toxic effects as histamine enters the bloodstream (Taylor, 1986). Putrescine and cadaverine can inhibit these enzymic reactions and therefore potentiate the toxicity of histamine (Eitenmiller *et al.*, 1980). No limits have been set in the Australian Foods Standards Code for putrescine and cadaverine. These amines may also be toxic in addition to their DAO and HMT enzyme inhibiting effects, which potentiate the toxicity of histamine. Biogenic amines have been implicated in food poisoning incidents, usually from the consumption of fermented foods like cheese, meat, fish products and wine (Silla,

1996). Biogenic amines in food are mainly formed by decarboxylation of the corresponding amino acids by microorganisms (Moreno-Arribas *et al.*, 2003). Putrescine the biosynthetic precursor of polyamines is produced by two pathways; one by the decarboxylation of ornithine and another by the decarboxylation of arginine to agmatine (Morris and Jorstad, 1970; Moreno-Arribas *et al.*, 2003).

The production of biogenic amines in fermented food products is due to the activity of bacteria. *Enterobacteriaceae* can be involved in the production of histamine, cadaverine and *Pseudomonas* in the production of putrescine (Edwards *et al.*, 1987). Tyramine and phenylethylamine can be produced by *Enterococcus*, *Carnobacterium divergens*, *Carnobacterium piscicola* and some strains of *Lactobacillus curvatus* (Straub *et al.*, 1994; Masson *et al.*, 1996; Roig-Sagues *et al.*, 1996).

2.4. Silage

Ensilation is defined as a conservation process in which acids added or produced inhibits pathogen growth (Shirai *et al.*, 2001). Fish silage is a liquefied fish biomass produced by mixing with acid. The chopped fish mass is mixed with acid solution and stirred several times daily, until liquefied. The proteolytic enzymes present in the fish waste material break down the protein to liquid silage and the acid added reduce the pH to prevent microbial spoilage (Martin, 1996 c). It can be stored for several months. The best acids used in the study were propionic acid and formic acid (Wiseman *et al.*, 1982; Rattagool *et al.*, 1980; Green *et al.*, 1983). Other mineral acids used were hydrochloric acid and sulphuric acid (Cervantes, 1979; Alvarez, 1972). Extensive study has been conducted by Raa and Gildberg (1982) in fish silage to increase productivity of seafood industry.

Another biological method of ensilation is fermentation using microorganisms. Bacterial silage making is based on natural fermentation by lactic acid bacteria (LAB), which ferment water-soluble carbohydrates (WSC) to organic acids, mainly lactic (LA), under anaerobic conditions. As a result, the pH

decreases, inhibiting detrimental anaerobes and so the moist forage is preserved. The process can be divided into four phases with distinct characteristics:

1. Aerobic, when air (oxygen) is still present between the plant particles and the pH is 6.0-6.5. These conditions enable continuation of plant respiration, protease activity and the activity of aerobic and facultative aerobic microorganisms such as fungi, yeasts and enterobacteria.
2. Fermentation, lasting several days to several weeks after the silage becomes anaerobic. LAB develop and become the predominant microbial population. Lactic and other acids are produced, and the pH decreases to 3.8-5.0.
3. Stable, when relatively few changes occur if air is prevented from entering the silo.

4. Feed out, when the silage is unloaded and then is exposed to air. This allows re-activation of aerobic microorganisms, mainly yeasts, moulds, bacilli and acetic acid bacteria, causing spoilage. (Weinberg and Muck, 1996).

During ensilation, lactic acid bacteria will convert the carbohydrate to lactic acid and lower the pH. The mixture will therefore be protected from microbial degradation, allowing proteolytic enzymes (proteases), present in the fish muscle or viscera, to break down protein tissues and produce a liquefied (hydrolysed) product. Most of the proteins are hydrolysed to short peptides, some of which may be further degraded to free amino acids (Martin, 1996 a). The final product silage can be used as an animal feed and fertilizer (Haard *et al*, 1985). Pathogenic bacteria such as coliforms and enterococci and spores such as those of *Clostridium botulinum* will be destroyed during the ensiling process (Raa *et al.*, 1983). The advantages in the use of lactic acid bacteria over the use of the acid in silage preparation includes avoiding the need to work with acids, simplicity of maintenance and reproduction of bacterial cultures, monetary savings by not having to purchase acids and a lower moisture level in the

product as a result of the lactic acid silage process, which facilitates drying of the product (Dong *et al.*, 1993).

Each phase of the ensiling process should be controlled in order to maintain silage quality. The sensitivity of the silage to spoilage at the various stages depends on the crop ensiled, the ensiling technology and silo type (Weinberg and Muck, 1996). Bacterial inoculants have advantages over chemical additives because they are safe (non-hazardous), easy-to-use, non-corrosive to farm machinery, do not pollute the environment, and were regarded as natural products. It is an essential component of feed ingredients especially in many Scandinavian countries. Advantages of using fish silage include efficient utilization of opportunity feedstuffs, decreased storage space and cost and good storage durability. In addition, fish silage lowers the pH of the diet, thus improving the hygienic quality of the feed. Low dietary pH will also reduce the pH of the urine and prevent the formation of bladder and kidney stones. Moreover, fish silage will affect feed consistency and reduce the need for added water in the feed. It will thus increase dietary dry matter and energy density per kg fed, which in return lowers feed consumption. The major drawback is low pH, which causes acidosis and affects the palatability.

3. MATERIALS AND METHODS

3.1. Raw Material- Shrimp Shell

Shrimp (*Metapenaeopsis dobsoni*) shell was obtained from the peeling shed of a shrimp processing plant located at Cochin, Kerala. The waste was brought to the laboratory within 3 hours of peeling and was minced in a meat mincer.

3.2. Jaggery

Procured from the local market in Cochin, Kerala

3.3. Chemicals

Analytical grade reagents supplied by BDH, Poole, England and Sigma chemical company, St.Louis, U.S.A. were used for the studies.

3.4. Starter Cultures

Lactobacillus plantarum, Lactobacillus brevis and Bacillus subtilis were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.5. Analytical Equipment

- Balance: Sartorius Electronic Balance, Germany
- Hot air oven: Beston hot air oven
- Glasswares: Borosil Glasswares
- pH meter: Cyberscan 510 pH meter. Euteoh Instruments, Singapore
- Spectrophotometer: Spectronic Genesys 5, Spectronic Instruments, Inc., USA
- Incubator: Thermo Scientific USA
- Autoclave: Obromax, India

- Muffle Furnace: Shikovi heatgen Technologies Pvt. Ltd.
- Brookfield DV-E Viscometer with ULA spindle (SMC- 0.64)
- Shimadzu Amino acid analyzer
- Nicolet AVATAR 360ESP FTIR Spectrometer
- Varian SpectrAA 220 Atomic Absorption Spectrometer.
- Schott Gerate AVS 410 intrinsic viscosity meter.

3.6. Methods

3.6.1. Fermentation experiments using *Lactobacillus plantarum*

3.6.1.1. Optimization of fermentation parameters for fermentation of shrimp shell using *Lactobacillus plantarum.*

3.6.1.1.1. Preparation of jaggery broth

The sucrose content of the jaggery was measured and accordingly different concentrations of jaggery solution, (10%, 15%,20%,25%,30%w/v)prepared were sterilized in an autoclave at 10 psi for 15 minutes.

3.6.1.1.2. Inoculum preparation

Commercial starter of *Lactobacillus plantarum* was maintained in MRS agar slopes at 4^0 C. The inoculum was prepared by adding a loopfull of cells to 10 ml MRS broth and incubated at 30^0C for 24 hrs Then 0.5ml of this was transferred to 100 ml of sterilized jaggery broth (10%,15%,20%,25%,30%w/v) prepared at different concentrations and incubated at 30^0C for 24 hrs.

3.6.1.1.3. Flask fermentation

Shrimp waste mixed with jaggery broth (1:1) prepared at different sugar concentrations (10%,15%,20%,25% and 30%w/v) were kept for fermentation for

21 days. Treatments were kept in triplicate and samples were drawn in 48 hrs interval for analysis. pH, Total titrable acidity, protein content, sugar concentration,proteolytic activity and microbial count of the protein liquor was measured. Ash and protein content of the fermented residue were estimated.Odour evaluation of the treatments were done by three panelists.

3.6.1.2. Fermentation of shrimp shell using *Lactobacillus plantarum*

3.6.1.2.1. Inoculum preparation

Commercial starter of *Lactobacillus plantarum* was maintained in MRS agar slopes at 4^0 C. The inoculum was prepared by adding a loopfull of cells to 10 ml MRS broth and incubated at 30^0C for 24 hrs. Then 0.5ml of this was transferred to 100 ml of sterilized 20% w/v jaggery broth and incubated at 30^0 C for 24 h

3.6.1.2.2. Flask fermentation

Minced shrimp waste (200 g) was thoroughly mixed with 24 hour incubated 20% w/v jaggery broth (200 ml) containing *Lactobacillus plantarum*. The inoculum prepared yielded a cell concentration of approximately 10^8 CFU/ml. They were kept in 2X3 factorial arrangement. Samples were mixed thoroughly and the initial pH was adjusted to 5.5, using acid, in the first 6 hours. Lactic acid was used in one container and dilute hydrochloric acid in another one and both were kept for fermentation for 15 days.

Liquor samples were taken at 48 hours intervals and analyzed for pH, total titrable acidity (TTA), proteolytic activity, sugar concentration and total lactobacillus count. After the completion of fermentation, sediments were removed, washed and estimated its ash, chitin and protein content. Amino acid composition, biogenic amines, alpha amino nitrogen content and total volatile base nitrogen of the protein liquor obtained after fermentation were studied.

3.6.1.3. Mild acid and alkali treatment of fermented residue to produce chitin

Solid fractions obtained after fermentation were treated with different concentrations of HCl (0.2, 0.5, 0.8 and 1 N) in 1:15 (solid: acid) ratio for 2 hours. The samples were then washed thoroughly and treated with different concentrations of NaOH (0.2, 0.4, 0.6, 0.8, 1 and 1.2 M) in 1:15 (solid: alkali) ratio for deproteinisation. Protein, ash and chitin contents were estimated. FTIR of the chitin samples were analysed. Mineral composition of chitin was also determined.

3.6.1.4. Chitosan preparation (Madhavan, 1992)

The purified chitin was boiled with 40% (w/v) NaOH till it gets deacetylated to chitosan. Chitosan formation was tested by its solubility in 1% acetic acid solution. Moisture content, ash content, FTIR spectra, viscosity, molecular weight, degree of deacetylation etc of the prepared chitosan were measured.

3.6.2. Fermentation experiments using *Lactobacillus brevis*

3.6.2.1. Optimization of fermentation parameters for fermentation of shrimp shell using *Lactobacillus brevis*

3.6.2.1.1. Preparation of jaggery broth

Same as 3.6.1.1.1

3.6.2.1.2. Inoculum preparation- Inoculum preparation of lactobacillusbrevis done as per the procedure 3.6.1.1.2.

Inoculum preparation was done by using commercial starter culture of *Lactobacillus brevis*. Method of preparation was the same as used in section 3.6.1.1.2

3.6.2.1.3. Flask fermentation

Flask fermentation of *Lactobacillus brevis* at different sugar concentrations were done as per the procedure described in section 3.6.1.1.3.

3.6.2.2. Fermentation of shrimp shell using *Lactobacillus brevis*

3.6.2.2.1. Inoculum preparation

Inoculum preparation using the commercial starter culture of *Lactobacillus brevis* was done adopting the procedure mentioned in section 3.6.1.2.1.

3.6.2.2.2. Flask fermentation

Minced shrimp waste (200 g) was thoroughly mixed with 24 hour incubated 20% w/v jaggery broth (200 ml) containing *Lactobacillus plantarum*. The inoculum prepared yielded a cell concentration of approximately 10^8 CFU/ml. They were kept in 2X3 factorial arrangement. Samples were mixed thoroughly and the initial pH was adjusted to 5.5, using acid, in the first 6 hours. Lactic acid was used in one container and dilute hydrochloric acid in another one and both were kept for fermentation for 17 days.

Liquor samples were taken at 48 hours intervals and analyzed for pH, proteolytic activity, sugar concentration and total lactobacillus count. After the completion of fermentation, sediments were removed, washed and estimated its ash, chitin and protein content. Amino acid composition, biogenic amines, alpha amino nitrogen content and total volatile base nitrogen of the protein liquor

obtained after fermentation were studied.

3.6.2.3. Mild acid and alkali treatment of fermented residue to produce chitin

Solid fractions obtained after fermentation were treated with different concentrations of HCl (0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 N) in 1:15 (solid: acid) ratio for 2 hours. The samples were then washed thoroughly and treated with different concentrations of NaOH (0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 M) in 1:15 (solid: alkali) ratio for deproteinisation. Protein, ash and chitin contents were estimated. FTIR of the chitin samples were analysed. Mineral composition of chitin was also determined.

3.6.2.4. Chitosan preparation

Same as 3.6.1.4

3.6.3. Fermentation experiments using *Bacillus subtilis*

3.6.3.1. Optimization of fermentation parameters for fermentation of shrimp shell using *Bacillus subtilis*

3.6.3.1.1. Preparation of jaggery broth

Same as 3.6.1.1.1

3.6.3.1.2. Inoculum preparation

Commercial starter of *Bacillus subtilis* was maintained in Nutrient agar slopes at 4^0C . The inoculum was prepared by adding a loopfull of cells to 10 ml nutrient broth and incubated at 30^0C for 24 hrs. Then 0.5ml of this was transferred to 100 ml of sterilized jaggery broth (10%, 15%, 20%, 25%, 30% w/v) prepared at different concentrations and incubated at 30^0C for 24 hrs.

3.6.3.1.3. Flask fermentation

Fermentation of Bacillus subtilis in different sugar concentrations were done as per the procedure cited in section 3.6.1.1.3

3.6.3.2. Fermentation of shrimp shell using *Bacillus subtilis*

3.6.3.2.1. Inoculum preparation

Commercial starter of *Bacillus subtilis* was maintained in Nutrient agar slopes at 4^0C. The inoculum was prepared by adding a loopfull of cells to 10 ml nutrient broth and incubated at 30^0C for 24 hrs. Then 0.5ml of this was transferred to 100 ml of sterilized 20% w/v jaggery broth and incubated at 30^0C for 24 h.

3.6.3.2.2. Flask fermentation

Minced shrimp waste (200 g) was thoroughly mixed with 24 hour incubated 20% w/v jaggery broth (200 ml) containing *Bacillus subtilis*. The inoculum prepared yielded a cell concentration of approximately 10^8 CFU/ml. They were kept in 2X3 factorial arrangement. Samples were mixed thoroughly kept for fermentation for 15 days.

Liquor samples were taken at 48 hours intervals and analyzed for pH, proteolytic activity and sugar concentration. After the completion of fermentation, sediments were removed, washed and estimated its ash, chitin and protein content.

3.6.3.3. Mild acid and alkali treatment of fermented residue to produce chitin.

Chitin production from the fermented residue was carried out as per the method elaborated in section 3.6.1.3

3.6.3.4. Chitosan preparation

Same as 3.6.1.4

3.7. Analytical Methods

3.7.1. Determination of moisture (A.O.A.C., 1975)

5-10 g sample was weighed into pre-weighed clean petri dishes. Dishes were placed in a hot air oven at 100±1° C for 6 hours. Dishes were cooled in a desiccator and weighed to a constant weight. Moisture expressed as percentage.

$$\% \text{ Moisture} = \frac{\text{Loss in weight x 100}}{\text{Weight of the sample}}$$

3.7.2. Determination of crude protein (A.O.A.C., 1975)

0.3 to 0.5 g of the moisture free sample was transferred into a digestion flask of 50 ml capacity. A few glass beads, a pinch of digestion mixture (8 parts K_2SO_4 & 1 part $CuSO_4$) and 20 ml concentrated sulphuric acid were added to the flask. It was digested over a burner until the solution turns colourless. The digest was transferred quantitatively into a 100 ml standard flask and made up to the mark. The 2 ml of well-mixed made-up solution was transferred to the reaction chamber of the Micro-Kjeldahl distillation apparatus, 2 drops of phenolphthalein indicator and 40% sodium hydroxide

were added till the indicator changes to pink. Distillation was done for 4 minutes and ammonia liberated was absorbed into 2% boric acid containing a drop of Taschiro's indicator. The amount of ammonia liberated was determined by titration with N/50 sulphuric acid. % Total nitrogen is calculated from the titre value.

Approximately 0.5 g of the sample is taken in digestion flask, add 10 ml 3% NaOH solution and heated in water bath for 30 minutes. Cooled, filtered through Whattmann 40 filter paper with sufficient washing with distilled water to get the sample free of alkali. The precipitate obtained on the filter paper is collected in the kjeldahl digestion flask, added a small amount of digestion mixture, 20 ml con. H_2SO_4, put some glass beads and kept for digestion in the chamber till the sample turns colourless. The clear solution is transferred to 100rnl standard flask and made up with distilled water. The 2 ml of the solution was transferred to the reaction chamber of the Micro-Kjeldahl distillation apparatus, one or two drops of phenolphthalein indicator and 40% sodium hydroxide were added till the indicator changes to pink. Distillation was done for 4 minutes. The liberated ammonia was absorbed into 2% boric acid containing a drop of Taschiro's indicator. The amount of ammonia liberated was determined by titration with N/50 sulphuric acid. % Chitin nitrogen is calculated from the titre value.

Crude protein was calculated according to the following formula. Crude protein was expressed as percentage

% Crude protein = (Total Nitrogen- Chitin Nitrogen) X 6.25

3.7.3. Determination of ash content (A.O.A.C. 1975)

About 2-3 g of the moisture free sample was transferred into a previously heated, cooled and weighed silica crucible. The sample was charred at low red heat. Then the crucible was placed in a muffle furnace at 550° C for about 6 hours until a white ash was obtained. Crucible was cooled in a desiccator and weighed. Ash content expressed as percentage.

$$\% \text{ Ash} = \frac{\text{Weight of residue x 100}}{\text{Weight of the sample}}$$

3.7.4. Measurement of pH (Adams et al., 1987)

pH of the liquor obtained during the fermentation was measured in 10 ml of sample diluted 1:10 with distilled water and pH measured using Cyberscan pH meter.

3.7.5. Measurement of Total Titrable Acidity (TTA) (Adams *et al.*, 1987)

Using the same homogenate prepared for the pH, TTA was measured by titrating against 0.1N NaOH to a final pH of 8.The percentage w/v lactic acid in the sample was calculated by multiplying the volume of alkali (ml) by a factor of 0.09. This assumes that all the acid present in the sample is lactic acid.

3.7.6. Estimation of sugar content (Hodge and Hofreiter, 1962).

Serial dilutions of protein liquor were made up to 3ml using distilled water and add 6ml anthrone reagent. Boil the sample for 3 minutes and cool and read the absorbance at 620nm keeping a blank. Absorbance of the standard glucose solution also were measured. Anthrone reagent was prepared by dissolving 200mg anthrone in 5ml distilled water and 95 ml concentrated sulphuric acid.

Concentration of sugar was expressed as mg %

3.7.7. Estimation of protein content (Lowry *et al.*, 1951)

Serial dilutions of the sample were prepared in different test tubes and standard BSA in the range of 20-100µg added and the total volume was made up to 1.0ml with water. The blank is taken with 1.0ml of water. Pipette 4.5ml of alkaline copper reagent was added to all the tubes and left at room temperature for 10minutes after which was added 0.5ml Folin's phenol reagent. The blue colour developed was read after 20 minutes at 640nm against the reagent blank, in a spectronic genesys UV spectrophotometer.

Protein values are expressed as gm protein per ml of the liquor

3.7.8. Determination of proteolytic activity (Wang and Chio, 1998)

The protein liquor was centrifuged at 10000 rpm for 20 min .0.2 ml of the supernatant was mixed with 2.5 ml 1% casein in phosphate buffer pH 7 and incubated for 10 minutes at 37^0 C. The reaction was stopped by adding 5 ml of 0.19 M TCA. The reaction mixture was centrifuged and the soluble peptide in the supernatant fraction was measured with tyrosine as standard.

1.0 ml of supernatant was made alkaline by 5.0 ml of 0.55 M sodium carbonate. Then pipette 0.5 ml of 1N Folin's reagent to the above solution in test tube. The optical density was measured at 660 nm after 30 min. at room temperature in Spectronic Genesys spectrophotometer.

Results were expressed as g Tyrosine/g protein.

3.7.9 Lactic acid bacterial count (de Man *et al.*, 1960)

10 ml of the sample diluted with 90 ml of normal saline, shaken well. Serial dilutions were made and poured 9 ml normal saline. Then pour plating method was used. Pipette 1ml of the sample from each test tube to two petri dishes and pour MRS agar over it. Then kept for incubation for 24 hours. Plates with 30-300 colonies were counted and expressed as CFU/ml.

3.7.10. Viscosity

Viscosity of 1% of solution was measured using Brookfield DV-E Viscometer with ULA spindle (SMC- 0.64) at 100 rpm. Viscosity was expressed in m Pa.S

3.7.11. Degree of deacetylation (Muzzarelli and Rocchetty, 1973)

1% w/v of chitosan solution was prepared in 1% v/v acetic acid. From this, 1 g of the solution was taken and made up to 100 ml with distilled water. Absorbance of this solution was taken at 200, 201, 202, 203 and 204 nanometers. Regression of these values was found out and from the b value

obtained degree of deacetylation was calculated. Degree of deacetylation was expressed in percentage (%)

3.7.12. Molecular weight (Rutherford and Austin, 1978)

For the determination of molecular weight, five dilutions of chitosan were prepared in 1% acetic acid. These solutions were run in Schott Gerate AVS 410 intrinsic viscosity meter. From the values obtained, intrinsic viscosity (η) was calculated. The Mark-Houwink equation relating to intrinsic viscosity with empirical viscometric constants $K=1.81 \times 10^{-3}$ cm^3/g and $a=0.93$ for chitosan was used to calculate the molecular weight (M) using the following equation:

$$[\eta]=KMa.$$

Molecular weight was expressed in Daltons (Da)

3.7.13. FTIR spectrum

Samples were mixed well with KBr using a mortar and pestle. This whole mixture was made into discs and FTIR spectra obtained with Nicolet AVATAR 360ESP FTIR Spectrometer. The average number of scans taken per samples were 16 in the spectral region 400 and 4000 cm^{-1}

3.7.14. Percentage of N-acetylation (Teng *et al.*, 2001)

From the FTIR spectra of the samples, absorbance at 1655 wavelength and 3450 obtained.% of N-acetylation = $(A_{1655}/A_{3450}) \times 115$. Here, A1655 and A3450 were the absorbance at 1655 cm^{-1} of the amide-I band as a measure of the N-acetyl group content and 3450 cm^{-1} of the hydroxyl band as an internal standard. The factor '115' denoted the value of the ratio of A1655 / A3450 for fully N-acetylated chitosan.

3.7.15. Preparation of TCA extract

10 ml of the protein liquor was mixed with 10% trichloroacetic acid and filtered the solution and made upto 100ml in standard flask.

3.7.16. Estimation of Alpha Amino Nitrogen (Pope and Stevens, 1939)

20ml of the TCA extract was taken and it was neutralized with 3Nsodium hydroxide using thymolphthalein indicator.the end product is the disappearance of the blue colour.30ml of the cupric phosphate suspension was added to this and allowed to stand fro 30mts and filtered to iodine flask.20ml of the filtrate was removed and 0.5ml of the glacial acetic acid and 2g of potassium iodide were added to it.Then titrate with 0.01N sodium thiosulphate until the solutiuon was pale yellow.then 1-2 ml of starch was added and titrated till it became colourless. Blank was also done in the same way.

Alpha amino nitrogen expressed as mg%

3.7.17. Estimation of total volatile base nitrogen (Conway, 1950)

1ml of supernatant from the TCA extract was pipetted to the outer chamber of Conway's microdiffusion unit.1ml of the standard sulphuric acid was pipetted to the inner chamber of the unit and 1ml of saturated potassium carbonate to the outer chamber and close the unit immediately.It was kept overnight.Acid in the central chamber was titrated against 0.01N Sodium hydroxide using Taschiro indicator. Blank was also done.

Total volatile base nitrogen expressed as mg%

3.7.18. Amino acid composition (Rajendra, 1987)

To 0.2 ml liquor sample in a heat stable test tube.,add 10ml 6N HCl and heat seal the tube after filling with pure nitrogen gas. Keep it at 110^0C for 24 hours for hydrolysis. When the hydrolysis completed, remove the contents quantitatively. Then filter the sample to a round bottom flask through Whatmann

filter paper No. 42 and wash the filter paper 2-3 times with distilled water. Flash evaporate the contents of the flask and dissolve the residue and make up to 10 ml with 0.05 M HCl.

Filter the sample thus prepared again through a membrane filter of 0.45 µm and inject 20 µl of this to an amino acid analyzer (HPLC- LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin i.e., styrene di vinyl benzene co polymer with sulphinic group. The column used was sodium type i.e., ISC- 07/S 1504 Na having a length of 19 cm and diameter 5mm.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A Chrompac recorder. The mobile phase of the system consists of two buffers, Buffer A and buffer B. A gradient system can be followed for the effective separation of amino acids. The oven temperature can be maintained at 60^0 C. The total run was programmed for 62 minutes. The amino acid analysis can be done with non-switching flow method and fluorescence detection after post–column derivatization with o-phthalaldehyde. In the case of proline and hydroxyl proline, imino group is converted to amino group with hypochlorite.

Amino acid standard (Sigma chemical Co., St. Lousis, USA) is also injected to HPLC to calculate the concentration of amino acids in the sample. Calibration of equipment using standards needs to be done before the start of analysis.

The standard and the sample were analyzed under identical conditions. The elution time of the amino acids of the sample was compared and identified with those of the standard.Quantification of amino acid was done by comparing the respective peak areas I n the chromatogram of the sample and the standard. The amino acid content was expressed as (g/100g crude protein).

3.7.19. Estimation of Tryptophan (Sastry and Tummuru, 1985)

Sample was hydrolyzed with 10 ml of 5% NaOH at 110^{0}C for 24 hours in a sealed tube with pure nitrogen. The hydrolysate was neutralized to pH 7.0 with 6 N HCl using phenolphthalein indicator. The volume was made up to 25 ml with distilled water. The solution was then filtered through Whatman filter paper no.42 and filtrate was used for estimation.

The test tube containing 4 ml of 50% H_2SO_4, 0.1ml of 2.5% sucrose and 0.1ml of 0.6% thioglycolic acid were added. These tubes were kept for 5minutes in water bath at 45-50^{0}C and cooled. The sample was then added to the test tubes. Standard tryptophan (10µg/ ml solution) was run in a similar way. The volume was made up to 5ml with 0.1N HCl and allowed to stand for 5 minutes for the development of colour. The absorbance was measured against a reagent blank at 500 nm in a spectrophotometer.

The tryptophan was expressed as (g/100g crude protein).

3.7.20. Estimation of minerals using Atomic Absorption Spectrophotometer (AOAC ,1980)

To 1g of the sample containing flask, 7 ml of nitric acid and perchloric acid (9:4) mixture was added, covered with a watch glass and left at room temperature over night. The sample was then digested using a microwave digester (Milestone ETHOS PLUS lab station Closed Vessel Microwave Digestion System). The completely digested samples were allowed to cool at room temperature, filtered (glass wool) carefully transferred into a clean 50 ml volumetric standard flask and then diluted to the mark with ultra pure water (Milli Q, Millpore). The digested samples were analyzed using Varian SpectrAA 220 Atomic Absorption Spectrometer equipped with a deuterium back ground corrector, for the determination of minerals viz sodium, potassium, calcium and magnesium.

Mineral content expressed in ppm.

3.7.21. *Bacillus subtilis* count (Aoyama *et al.*, 2005)

Sample was serially diluted in sterile saline (0.85% NaCl). Each diluted plate was poured into duplicate plates of standard agar. After cultivating the plates 24 h at 35^0 C, the viable colonies were counted. Plates with 30-300 colonies were counted and expressed as CFU/ml.

3.8. Statistical Analysis

Results were expressed as mean ± S.D. Univariate analysis of variance was carried out and the statistical comparisons were done with Duncan's test using a statistical package program (SPSS 10.0 for Windows). Five trials were done for each parameter.

4. RESULTS AND DISCUSSIONS

4.1. Fermentation studies with *Lactobacillus plantarum*

4.1.1. Optimisation of fermentation parameters

4.1.1.1. Inoculum quantity standardization

Type of the starter culture and its quantity is a very important factor that affects the efficiency of fermentation. Inoculation of suitable lactic acid bacteria ensures rapid acidification and eventual predominance of desired microflora, which is able to conduct ensilation (Shirai *et al.*, 2001). *Lactobacillus plantarum* was used as the starter culture for the study because the species meet most of the criteria presented by Whittenbury (1961). A starter culture of proper lactic acid bacteria has to be added for fermentation because they are present in low numbers in its biomass in the order 10-10^4/ g (Knachel, 1981) and many reports were available regarding the fermentation with lactic acid bacteria (Cira *et al.*, 2002, Shirai *et al.*, 2001). Weinberg and Muck (1996) reported that minimum inoculation rates of 10^5-10^6 viable cells /g are required to outgrow the epiphytic lactic acid bacteria and to become the predominant population in the ensilage. The use of large number of cells as inoculum or as starter culture helps to discourage the colonization of undesired organism by the mechanism of antagonism (Holzapfel *et al.*, 1995).

In the present study, 0.5 ml of inoculum from MRS broth was inoculated to different concentrations of jaggery broth prepared (10%, 15%, 20%, 25% and 30% w/v) and achieved an approximate growth of 10^8 CFU/g after 24 hrs of incubation at 30^0 C. These broths were added to minced shrimp waste in 1:1 ratio for fermentation. So, the inoculum rate in this study was 1X10^8 CFU/g of wet shrimp waste. This is in line with the report by Evers and Caroll (1996).

4.1.1.2. Optimisation of sugar concentration and fermentation period

4.1.1.2.1. Changes in pH and Total Titrable Acidity during fermentation

The changes in pH were closely reflected by changes in total titrable acidity as shown in Table 4.1. The decrease in the concentration of sugar with

Table 4.1. Changes in pH and Total Titrable Acidity-TTA (% w/v) on treatment of shrimp shell with *Lactobacillus plantarum* at different jaggery concentrations with fermentation time

Days	Jaggery concentration (%)									
	10		15		20		25		30	
	pH	TTA	pH	TTA	pH	TTA	pH	TTA	pH	TTA
0	-	-	-	-	-	-	-	-	-	-
2	4.59±0.21	1.91±0.13	4.54±0.31	1.96±0.11	4.37±0.25	2.13±0.18	4.31±0.26	2.19±0.14	4.30±0.28	2.20±0.16
4	4.23±0.23	2.27±0.19	4.16±0.28	2.18±0.16	4.16±0.24	2.34±0.19	4.11±0.25	2.39±0.18	4.09±0.25	2.41±0.12
6	4.12±0.18	2.38±0.21	4.08±0.17	2.38±0.16	4.08±0.18	2.38±0.16	4.07±0.21	2.43±0.11	4.05±0.21	2.45±0.15
8	5.13±0.21	1.37±0.11	4.43±0.26	2.07±0.19	4±0.23	2.5±0.16	3.97±0.18	2.63±0.10	3.96±0.29	2.54±0.15
10	5.25±0.32	1.25±0.10	5.08±0.32	1.42±0.12	4.01±0.20	2.49±.0.15	4.0±0.19	2.5±0.09	4.0±0.18	2.5±0.18
12	5.46±0.35	1.04±0.09	5.18±0.25	1.32±0.13	4.48±0.28	2.02±0.11	4.46±0.26	2.04±0.01	4.52±0.32	1.98±0.11
14	5.65±0.38	0.85±0.08	5.36±0.39	1.14±0.09	5.22±0.32	1.28±0.07	5.20±0.31	1.3±0.08	5.18±0.38	1.32±0.09
16	5.78±0.31	0.72±0.06	5.58±0.31	0.92±0.05	5.48±0.31	1.02±0.05	5.42±0.28	1.08±0.09	5.40±0.31	1.1±0.07
18	5.89±0.42	0.61±0.06	5.76±0.25	0.74±0.04	5.67±0.3	0.83±0.02	5.53±0.36	0.97±0.06	5.50±0.3	1.0 ±0.07
20	6.23±0.36	0.27±0.01	6.01±0.21	0.49±0.02	5.94±0.42	0.56±0.03	5.98±0.35	0.52±0.03	5.95±0.27	0.55±.0.06

fermentation together with declining trend of pH value have a clear indication of conversion of added jaggery to acidic end products (Fig. 4.2). During fermentation, the bacteria utilizes the available sugar and produces acid and thereby the pH declined during the initial days. Microbial count was found to increase with respect to the increment in concentration of sugar broth (Table 4.3). Accordingly, acid production and pH declination were also varied (Legaretta *et al.*, 1996). When available sugar for microbial growth depleted, acid production decreased. It has strong correlation with odour evaluation results also (Fig. 4.1). In all the samples, pH first decreased and then started to increase. The rise in pH in later stages was mainly due to the depletion of sugar for lactic acid production. The buffering capacity of the calcium from the shell solubilised into the media (Zakaria *et al.*,1998) and nitrogenous compounds released by the proteolytic activity of the endogenous and microbial enzymes were also responsible for the increase in pH values of the protein liquor (Shirai *et al.*, 2001). *Lactobacillus plantarum*, which is homofermentative in nature, convert the sugar almost completely to lactic acid (Leroy and de Vuyst, 2003) and assuming that all the acid produced in the sample as lactic acid, total titrable acidity was measured (Adams *et al.*, 1987).

4.1.1.2.2. Odour Evaluation Results

The number of days the samples remained in good condition based on odour evaluation results were presented in the Fig. 4.1.The pH decrease, which is a clear indication of acid produced, helps in preventing the growth of spoilage organisms (Zakaria *et al.*, 1998) and also different antimicrobial compounds (de Vuyst and Vandamme, 1994; Hugas and Monfort, 1997) like H_2O_2, acetoin and bacteriocins inhibit spoilage microorganisms. The inhibitory activity of lactic acid is higher at low pH values due to the diffusion through the membrane in a non-ionised form and subsequently dissociating within the cell causing the disruption of some metabolic processes (Shirai *et al.*, 2001, Silliker *et al.*, 1980). During fermentation, the break down of proteins to small units directly affected the water activity essential for lactic acid bacteria growth (Shirai, 1999; Horner, 1997) and this reduction in water activity was responsible for the signs of putrefaction noticed in the samples (Shirai *et al.*, 2001). The silage at pH 4.5 and above is always susceptible to spoilage caused by *Clostridium botulinum*, *Staphylococcus aureas* and fungus (Anon, 1971). In higher concentrated sugar broths there was

Fig. 4.1. Number of days the fermented samples remained in acceptable condition on *Lactobacillus plantarum* treatment.

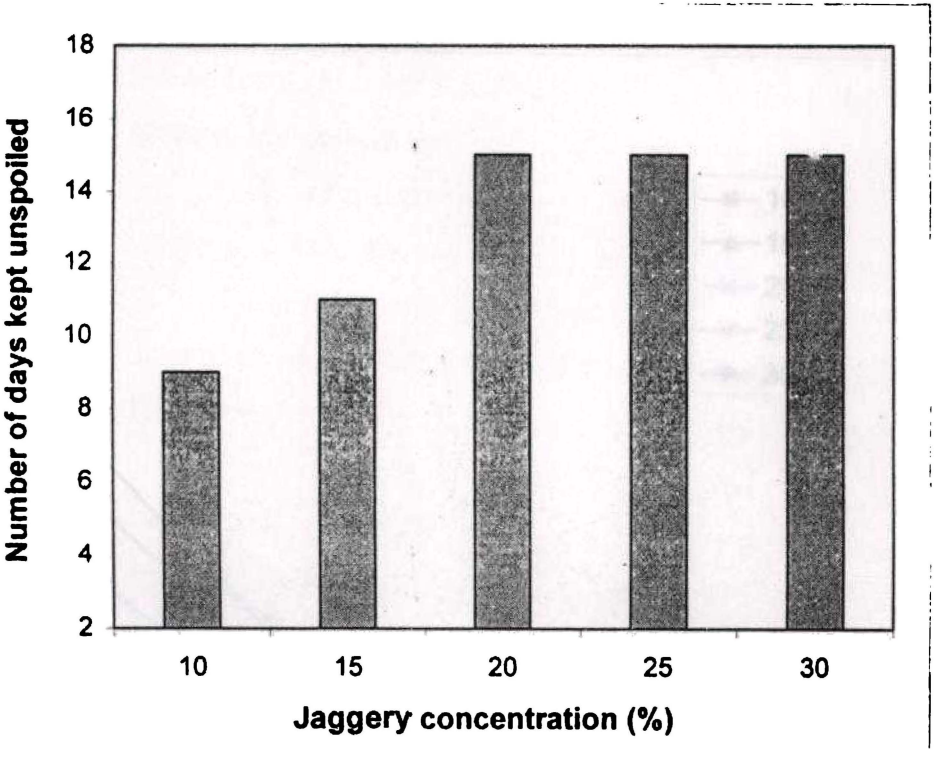
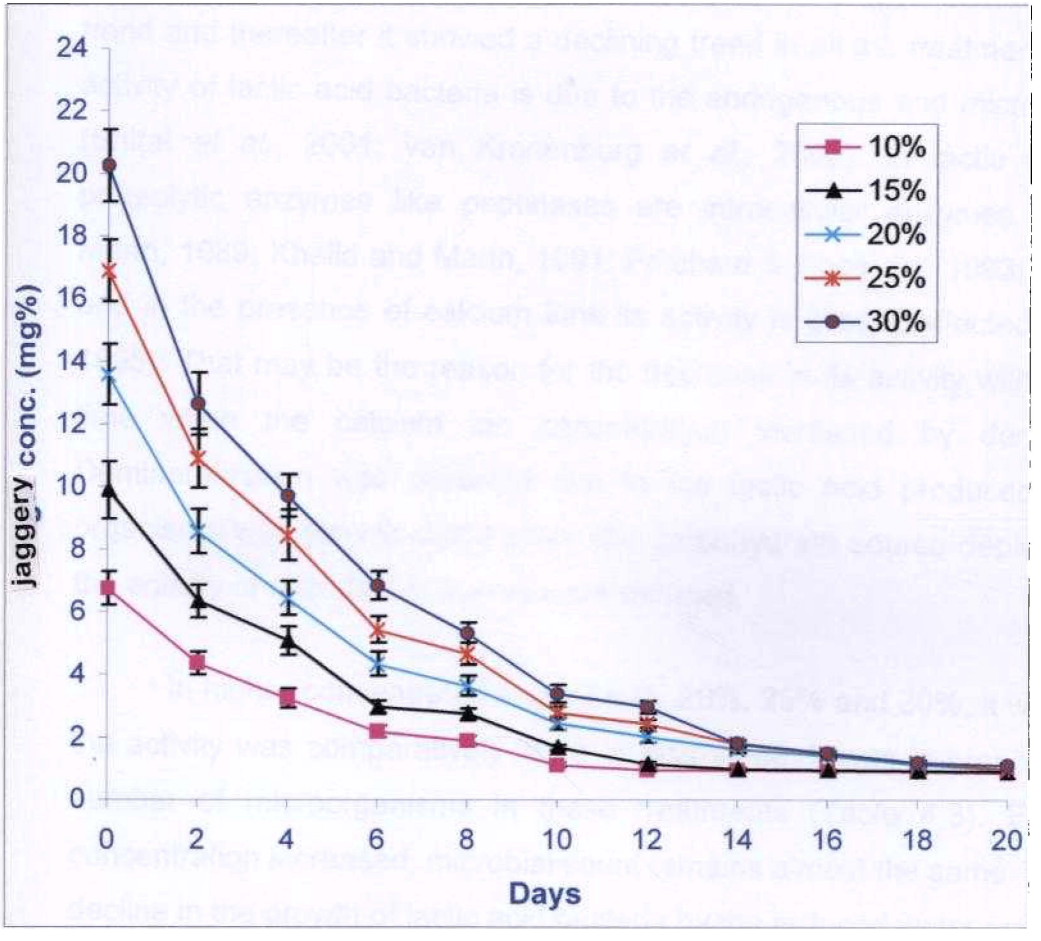


Fig. 4.2. The extent of utilisation of**Jaggery**in fermentation trials in *Lactobacillus plantarum* fermentation of shrimp shell



no improvement in their shelf life, due to reduced water activity by the high concentration of metabolisable sugar.

4.1.1.2.3. Changes in proteolytic activity

From Table 4.2, it was clear that, proteolytic activity showed an increasing trend and thereafter it showed a declining trend in all the treatments. Proteolytic activity of lactic acid bacteria is due to the endogenous and microbial enzymes (Shirai *et al.*, 2001; van Kranenburg *et al.*, 2002). In lactic acid bacteria, proteolytic enzymes like peptidases are intracellular enzymes (Kamaly and Marth, 1989; Khalid and Marth, 1991; Pritchard & Coolbear, 1993; Visser, 1993) and in the presence of calcium ions its activity is greatly affected (Dako *et al.*, 1995). That may be the reason for the decrease in its activity with fermentation time when the calcium ion concentration increased by demineralization. Demineralization was occurred due to the lactic acid produced. Number of organisms was also declined when the carbohydrate source depleted and thus the activity of microbial enzymes were reduced.

In higher concentrated sugar broth, 20%, 25% and 30%, it was found that the activity was comparatively more or less same. It was in proportion with the number of microorganisms in these treatments (Table 4.3). Even if sugar concentration increased, microbial count remains almost the same. This is due to decline in the growth of lactic acid bacteria by the reduced water activity.

4.1.1.2.4. Changes in microbial count.

The changes in bacterial count was depicted in the Table 4.3. Bacterial count showed an increasing trend in all the samples. It utilised the available sugar for its growth. It was clearly reflected in the changes of sugar concentration in all treatments. When the sugar substrate declined, counts of organisms were also decreased. When the metabolisable sugar concentration increased more than 10%, water activity reduced and the lag phase of lactic acid bacteria extended (Shirai *et al.*, 2001). This may be the reason for not much

Table 4.2. Changes in Proteolytic activity (g tyrosine/g protein) on treatment of shrimp shell with *Lactobacillus plantarum* at different jaggery concentrations with fermentation time

Days	**Jaggery**concentration (%)				
	10	15	20	25	30
0	-	-	-	-	-
2	6.01±0.32	6.32±0.42	6.53±0.36	6.58±0.39	6.59±0.45
4	6.53±0.36	6.61±0.48	6.89±0.39	6.91±0.31	6.95±0.42
6	6.98±0.41	7.32±0.48	7.81±0.48	7.85±.0.45	7.86±0.49
8	6.42±0.46	7.12±0.51	8.91±0.48	8.95±0.46	8.96±0.35
10	6.01±0.38	6.09±0.38	10.49±0.41	10.53±0.45	10.53±0.47
12	5.82±0.32	5.89±0.48	9.41±0.40	9.52±0.54	9.54±0.39
14	5.69±0.35	5.78±0.42	7.72±0.52	7.86±0.51	7.99±0.41
16	5.42±0.46	5.58±0.38	6.71±0.39	6.78±0.39	6.82±0.48
18	5.33±0.45	5.48±0.29	5.95±0.45	5.96±0.45	5.98±0.42
20	5.28±0.41	5.33±0.26	5.63±0.25	5.62±0.42	5.64±0.46

Table 4.3. Changes in microbial count (CFU/ml) on treatment of shrimp shell wit *Lactobacillus plantarum* at different jaggery concentrations wit fermentation time.

Days	Jaggery concentration (%)				
	10	15	20	25	30
0	$1.96 X10^{8}$	$2.11 X10^{8}$	$2.19 X10^{8}$	$2.20 X10^{8}$	$2.20 X10^{8}$
2	$2.45 X10^{9}$	$2.53X10^{9}$	$2.61X10^{9}$	$2.63X10^{9}$	$2.68 X10^{9}$
4	$2.55 X10^{9}$	$2.68X10^{9}$	$2.73 X10^{9}$	$2.75X10^{9}$	$2.76X10^{9}$
6	$2.73 X10^{8}$	$2.96 X10^{8}$	$2.86X10^{9}$	$2.88X10^{9}$	$2.89X10^{9}$
8	$1.82 X10^{8}$	$2.58 X10^{8}$	$2.93X10^{9}$	$2.96X10^{9}$	$2.97 X10^{9}$
10	$0.95 X10^{8}$	$1.26 X10^{8}$	$2.98 X10^{9}$	$2.99X10^{9}$	$2.99X10^{9}$
12	$1.75 X10^{7}$	$2.74 X10^{7}$	$2.84 X10^{8}$	$2.87 X10^{8}$	$2.89 X10^{8}$
14	$0.86 X10^{7}$	$2.18 X10^{7}$	$1.82X10^{8}$	$1.87X10^{8}$	$1.88 X10^{8}$
16	$0.35 X10^{7}$	$0.69 X10^{7}$	$1.65 X10^{8}$	$1.65X10^{8}$	$1.68 X10^{8}$
18	$2.10 X10^{6}$	$2.80 X10^{6}$	$2.46 X10^{7}$	$2.48X10^{7}$	$2.47X10^{7}$
20	$1.72 X10^{6}$	$2.40 X10^{6}$	$1.12 X10^{7}$	$1.16 X10^{7}$	$1.18 X10^{7}$

variation found in the microbial count in the high concentrated sugar broth (20%, 25% and 30%).

4.1.1.2.5. Type of sugar

As shrimp waste is poor source of fermentable sugars, some sources of sugars are to be added to it for bacterial fermentation. Different sources like sucrose, lactose, whey powder, glucose, molasses and cassava starch at different concentration were tried by many authors (Evers and Caroll, 1996; Cira *et al.*, 2002; Rao *et al.*, 2000; Oyedapo *et al.*, 1997). In the present study, jaggery was chosen as the carbohydrate source because it assisted the fermentation process, relatively inexpensive and can be used as a good medium for lactic acid bacteria growth, which will reduce the cost of the media and has high potential consumer acceptance by the seafood and livestock industry.

From our studies it was found that jaggery can be used as a substitute for MRS media to attain a growth of 10^8 CFU/g in 24 h incubation time and thus the cost of media can be reduced. Jaggery is a cheap source of sucrose and sucrose is reported to be an ideal carbohydrate source for lactic acid bacterial fermentation (Cira *et al.*, 2002)

4.1.1.2.6. Optimization of sugar concentration

Quantity of sugar influences directly the fermentation and earlier studies showed that minimum amount of glucose for successful fermentation was 5% w/v (Hassan and Heath, 1986; Van and Heydenrych, 1985). For the fermentation of shrimp waste, when glucose concentration was lower than 7.5%, pH was not reduced below 5.6 (Cira *et al.*, 2002). In the present study it was found that organism utilized about 45% of sucrose for inoculum development during its 24 h incubation period and only remaining sucrose content was available for fermentation experiment. Lactic acid bacteria utilise sugar and produces acid during fermentation. This was clearly reflected in the figure (Fig. 4.2) showing changes of sugar concentration during fermentation. At the end of fermentation, in all the treatments it was found that about 86-95% of sucrose was utilized

fermentation (Fig. 4.2). During the last spoilage days, the reduction in sugar concentration may be its utilization by spoilage microorganisms.

The selection criterion to find optimum sugar concentration in the present study was percentage of deprotenisation from shrimp shell (Table 4.4). By carrying out the statistical tests with SPSS it was found that, there was significant difference in the extent of deprotenisation of samples from 10%, 15% and 20% jaggery broth ($P<0.05$). But in the samples obtained from 20%, 25% and 30%, there were no significant difference in the percentage of deprotenisation. About 72.73% of protein was removed from shrimp shell at the end of fermentation. So, 20% w/v jaggery broth was selected for the study to reduce the cost.

4.1.1.2.6. Optimisation of fermentation period

For optimizing the fermentation period, odour evaluation results (Fig. 4.1) and other parameters like pH changes (Table 4.1), extent of utilization of sugar (Fig. 4.2), changes in proteolytic activity (Table 4.2), extent of deprotenisation (Table 4.4) etc. were considered.

According to odour evaluation results, the low concentrated sugar broth (10% and 15%) showed signs of spoilage on 8^{th} day and 10^{th} day respectively when the pH started rising. The high concentrated sugar broths 25% and 30% showed spoilage signs on same day as that of 20% sugar broth. It may be due to the reason that when the sugar concentration increased beyond 10% (amount of sugar available for fermentation), water activity was reduced due to the large water binding substance sucrose. The reduced water activity affected the growth of lactic acid bacteria and spoilage bacteria started their activity (Shirai *et al.*, 2001).

4.1.2. Fermentation of shrimp shell using *Lactobacillus plantarum* for the production of chitin and chitosan

4.1.2.1. A comparative study - The initial pH of the shrimp shell adjusted with an organic acid (Lactic acid) and inorganic acid (HCl).

Table 4.4. Percentage of deprotenisation of shrimp shell on fermentation with *Lactobacillus plantarum* at different jaggery concentrations.

Days	Jaggery concentration (%)				
	10	15	20	25	30
0					
2	17.52±1.12[a]	19.62±1.25[a]	22.19±1.39[a]	22.51±1.69	22.92±1.25
4	24.63±1.62[a]	28.77±1.52[a]	34.35±1.75[a]	34.60±1.48	34.92±1.63
6	37.81±1.69[a]	40.34±2.61[a]	44.09±2.14[a]	44.13±2.10	44.42±2.15
8	51.46±2.24[a]	53.52±2.84[a]	62.7±3.51[a]	62.91±3.81	63.00±5.81
10	62.98±2.21[a]	64.63±3.12[a]	67.69±4.11[a]	67.82±5.32	68.83±5.01
12	63.24±2.86[a]	65.12±2.59[a]	70.36±5.84[a]	70.61±4.18	70.96±4.52
14	63.37±2.91[a]	65.65±2.87[a]	72.66±5.98[a]	72.84±4.52	72.66±4.12
16	63.64±2.54[a]	65.83±1.69[a]	72.69±4.62[a]	72.72±5.02	72.80±4.82
18	63.81±3.30[a]	65.92±3.29[a]	72.68±3.96[a]	72.73±4.22	72.81±5.36
20	63.96±3.52[a]	66.26±3.12[a]	72.83±2.85[a]	72.93±4.15	72.93±4.59

[a] $P<0.05$ significantly different compared with different jaggery concentrations

4.1.2.1.1. Changes in pH and Total titrable acidity (TTA)

Changes in pH and TTA values were shown in Table 4.5. In lactic acid treated samples, reduction in pH and TTA increment were found more as compared to samples treated with HCl. It was found that pH first declined and then started increasing as fermentation proceeded supported by the decrease in residual carbohydrate (Fig. 4.3) in the fermenting media for bacterial growth.

During fermentation of shrimp shell waste, a general decrease in pH and increase in total titrable acidity with a later rise in pH and reduction in total acidity were noticed (Teniola and Odunfa, 2001). Fermentative bacteria produce acid, utilizing the available carbohydrate source and thus reduce the pH, which prevents the spoilage microorganism (Legaretta *et al.*, 1996) and that acid causes the demineralization of shellfish waste to produce chitin. Homofermentative lactic acid bacteria convert the available sugar source almost completely to lactic acid via pyruvate to produce energy (Leroy and de Vuyst, 2003).

Total titrable acidity was measured on the assumption that all the acid produced in the sample as lactic acid (Adams *et al.*, 1987). When the available sugar substrate for bacterial growth depleted, pH value increases and total titrable acidity value started to decrease. The increase in pH might be explained by the glucose depletion preventing lactic acid production, buffering capacity of the solubilised calcium and also due to the nitrogenous compounds produced by the proteolytic activity of endogenous and microbial enzymes (Shirai *et al.*, 2001). Prolonged low pH values can be maintained if continuous calcium removal is practiced (Cira *et al.*, 2002). The silage at pH 4.5 and above is always susceptible to spoilage caused by *Clostridium botulinum*, *Staphylococcus aureas* and fungus (Anon, 1971).

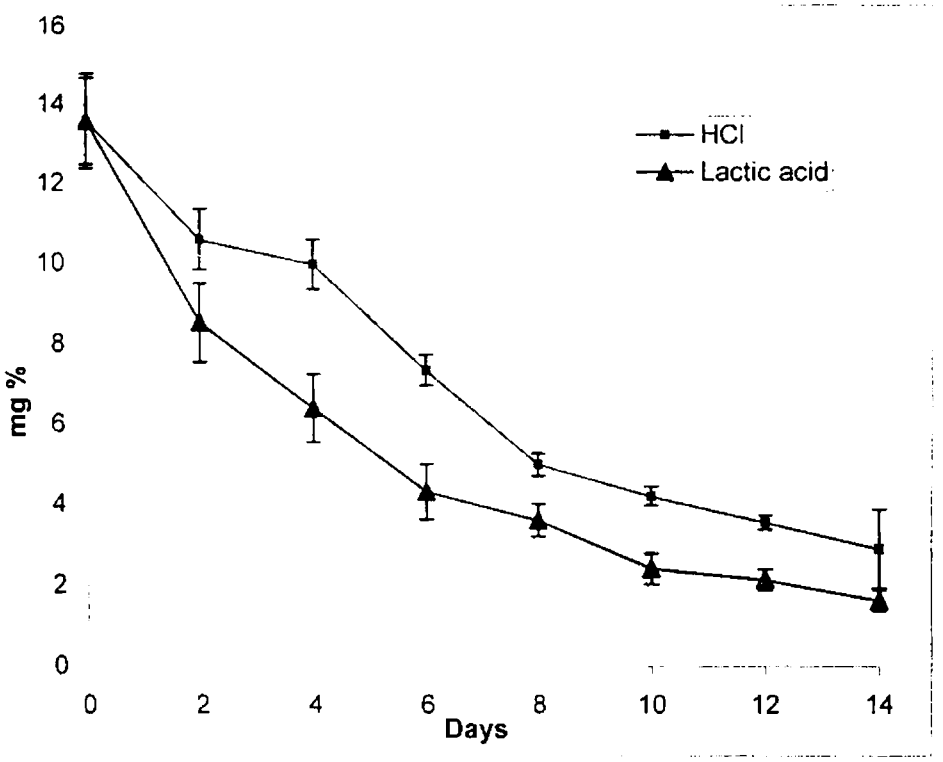
4.1.2.1.2. Changes in sugar concentration

About 92% of glucose was utilized in lactic acid treated samples and only 87% was used in HCl treated samples at the end of the fermentation (Fig. 4.3).

Table 4.5. Changes in pH and Total Titrable Acidity-TTA (% w/v) of the hydrochloric acid and lactic acid treated ensilage of *Lactobacillus plantarum* fermentation study

DAYS	HCl		Lactic acid	
	pH	TTA	pH	TTA
0	-	-	-	-
2	4.58±0.21	1.92±0.08	4.39±0.32	2.11±0.17
4	4.47±0.24	1.97±0.09	4.18±0.26	2.32±0.18
6	4.3±0.31	2.2±0.11	4.1±0.26	2.4±0.19
8	4.15±0.22	2.35±0.12	4±0.24	2.5±0.21
10	4.26±0.27	2.24±0.11	4.14±0.25	2.34±0.17
12	4.25±0.26	2.25±0.12	4.21±0.27	2.29±0.13
14	5.16±0.35	1.34±0.08	5.1±0.36	1.6±0.08

Fig. 4.3. Changes injaggeryconcentration of the hydrochloric acid and lactic acid treated ensilage of *Lactobacillus plantarum* fermentation study



The rate of utilization of sugar in lactic acid treated samples was more than hydrochloric acid treated samples in proportion with the microbial count.

4.1.2.1.3. Changes in bacterial count

Table 4.6. depicts the changes in bacterial count. It was found that bacterial count first increased and then decreased as fermentation time proceeded. In organic acid treated samples, rate of growth of bacteria was found to be more than HCl treated samples. It was found that changes in bacterial count have good correlation with changes in sugar concentration (Fig. 4.3). When the available sugar concentration for the bacterial growth decreased, microbial count also declined.

4.1.2.1.4. Changes in proteolytic activity

There was a steep increase in proteolytic activity upto 8 days and then started decreasing (Table 4.7). These results were in line with the degree of deproteinisation (Table 4.8). In lactic acid treated samples, comparative activity is found to be more than HCl treated one. This was in relation with the number of microorganisms (Table 4.6). Deprotenisation of shellfish waste also occurs during fermentation. It can be due to proteolytic activity of endogenous and microbial enzymes (van Kranenburg *et al.*, 2002). Lactic acid bacteria have a complex proteolytic system capable of converting protein to the free amino acids and peptides necessary for growth and acid production (Law and Haandrikman, 1997). We found that proteolytic activity of lactic acid bacteria showed an increasing trend up to 8th day of fermentation and there after it decreased. It was clearly supported by the trend of bacterial count and change in glucose concentration. Peptidases of lactic acid bacteria are mostly intracellular enzymes. (Kamaly and Marth, 1989; Khalid and Marth, 1991; Pritchard and Coolbear, 1993; Visser, 1993) When demineralization takes place, the amount of calcium ions in the ensilage increases and activity of peptidases decreases. (Dako *et al.*, 1995) That may be one of the reasons for the decrease in proteolytic activity after 9th day of fermentation. After fermentation of shell waste, the shell residue can be effectively converted to chitin and the protein liquor

Table 4.6. Changes in bacterial count (CFU/ml) of the HCl treated and lactic acid treated ensilage of *Lactobacillus plantarum* fermentation study

Days	HCl	Lactic acid
0	$2.13X10^8$	$2.18 X10^8$
2	$1.94X10^9$	$2.67 X10^9$
4	$1.56 X10^9$	$2.54 X10^9$
6	$1.14 X10^9$	$2.1 X10^9$
8	$2.57 X10^8$	$2.86 X10^8$
10	$2.40 X10^8$	$2.65 X10^8$
12	$2.12 X10^8$	$2.44 X10^8$
14	$1.81 X10^8$	$1.99 X10^8$

Table 4.7. Changes in proteolytic activity (g tyrosine/g protein) of the HCl treated and lactic acid treated ensilage of *Lactobacillus plantarum* fermentation trials.

Days	HCl	Lactic acid
0	-	-
2	5.43±0.29	6.83±0.42
4	6.38±0.35	7.79±0.48
6	6.99±0.38	8.99±0.56
8	7.78±0.46	10.63±0.75
10	6.59±0.41	9.31±0.59
12	6.06±0.42	7.71±0.42
14	5.86±0.39	6.32±0.42

Table 4.8. Percentage of protein and ash removed from the shell during fermentation with *Lactobacillus plantarum*

Days	HCl treated		Lactic acid treated	
	%of protein removed	%of ash removed	%of protein removed	%of ash removed
0	-	15.2±0.82	-	7.5±0.53
2	18.5±0.86	23.2±1.53	22.6±1.39	21.6±1.28
4	28.4±1.24	34.9±1.82	34.1±1.75	33.5±1.78
6	39.1±1.95	47.6±2.15	43.8±2.14	50.8±2.54
8	57.7±3.45	64.2±4.62	62.9±3.51	65.9±4.13
10	63.3±3.96	67.3±4.23	67.2±4.11	69.3±4.58
12	67.1±4.13[a]	69.7±5.31[b]	70.8±5.84[a]	73.8±6.14[b]
14	70.6±5.82[a]	71.8±5.94[b]	72.8±5.98[a]	76.5±6.47[b]

[a] $P<0.05$ significantly different in comparison of different acid treatments

[b] $P<0.01$ significantly different in comparison of different acid treatments

obtained can be used as an ingredient in animal feed (Meyers and Benjamin, 1987).

4.1.2.1.5. Percentage of deprotenisation and demineralisation

Percentage of protein removed showed a steep increase upto 8th day and thereafter slow trend of increment was noticed in all the samples (Table 4.8). This was well in coincidence with their trend in proteolytic activity. In HCl treated samples, about 70.6% of protein and in lactic acid treated samples 72.8% of protein was removed at the end of fermentation. There was a greater extent of demineralization noticed in HCl treated samples on the first day; but at the end of fermentation, about 76.5% of ash was removed in lactic acid treated samples and 71.8% in HCl treated samples (Table 4.8). It was found that there was a steep increase in percentage of demineralization upto 9th day of fermentation and thereafter it was slow down.

In the present study, lactic acid and dilute HCl were used for initial pH adjustment because without adjusting the initial pH, it took more than 5 hours for the lactic acid bacteria to convert glucose to lactic acid and during this delay spoilage occurred due to putrefying enzymes active at neutral or higher pH (Rao *et al.*, 2000). In HCl treated samples, a greater extent of demineralization was observed on the zeroth day and in the following days its value is less compared to lactic acid treated samples. It can be due to the greater strength of HCl than lactic acid by which it efficiently removed minerals on the zeroth day. But on following days, lactic acid treated samples showed greater degree of demineralization because number of colonies of lactic acid bacteria and simultaneously acid production was found to be more in this than HCl treated one. This is because when HCl is used for initial pH adjustment, it causes the decline in the number of organisms and is clearly supported by the data of microbial count (Table 4.6). As fermentation proceeds, pH reduction, total titrable acidity value and decrease in sugar concentration were dependant on the bacterial count.

Out of two different acids used, lactic acid treated samples achieved the

Out of two different acids used, lactic acid treated samples achieved the highest deproteinisation than hydrochloric acid treated samples ($P<0.05$). The difference is due to the number of microorganisms (Table 4.6) and its proteolytic activity. The less bacterial deproteinisation of shrimp shell, when treated with HCl, may be due to the destruction of protein that is required to induce protease enzyme, which is line with a previous study (Inayara *et al.*, 2005). Earlier studies also confirm the deprotenisation of shell fish waste by fermentation with lactic acid bacteria (Shirai *et al.*, 1997; Cira *et al.*, 2002). Differences in the extent of deprotenisation can be due to the method used, geographic source, species of shrimp waste, source of carbohydrate etc.

4.1.2.2. Mild acid and alkali treatment of the fermented residue to produce chitin.

A comparison of protein and ash content in the raw material and fermented shell residue is given in Table 4.9. In our study, maximum amount of minerals and protein were removed from lactic acid treated samples than HCl treated samples at the end of the fermentation.

The shrimp waste residue obtained after fermentation can be converted to chitin. The optimal concentrations of acid and alkali required to covert chitin depends on the residual protein and ash content which in turn dependent on the nature of fermentation, type of inoculum, starter culture, carbohydrate source, fermentation time etc (Shahidi and Synowiecki, 1991; No *et al.*, 1989; Cira *et al.*, 2002). Chitin was prepared from the fermented residue by chemical method. Considering the residual protein and ash content in fermented residues, different trials of chemical treatment were carried out to bring the protein and ash content within the permissible limit.

Percentage of ash content in the residue after chemical treatment is depicted in Fig. 4.4. From the figure it is clear that treatment with 0.8 N HCl produces chitin with ash content less than 1%, which is the permissible limit for good quality chitin (Tolaimate *et al.*, 2003), in both HCl treated and lactic acid treated samples. So, 0.8 N HCl was used for chitin preparation.

Table 4.9. Comparison of protein and ash content in the raw material and fermented shell residue of *Lactobacillus plantarum* fermentation

Particulars	% of protein content	% of ash content
Raw material	37.63±2.13	18.62±0.98
HCl treated	11.06±0.95	5.25±0.49
Lactic acid treated	10.23±0.86	4.37±0.36

Fig. 4.4. Percentage of ash content in the fermented residue on treatment with different concentrations of acid.

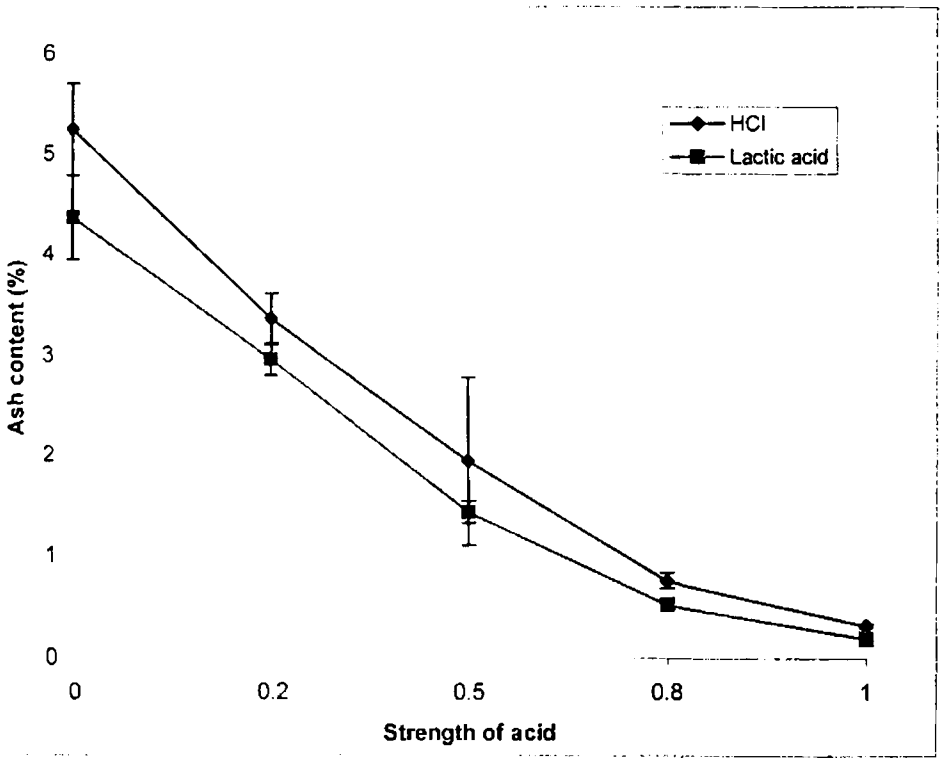


Fig. 4.5 shows the percentage of protein content in the fermented residue after chemical treatment. When the residue was treated with 1 M NaOH, protein content of the residue was reduced to less than 1%, which is the permissible protein content of standard chitin. So, deproteinisation was conducted with 1 M NaOH for the conversion of residue to chitin. Chitosan was prepared from this chitin as per the method described by Madhavan (1992).

4.1.2.3. Characteristics of the chitin and chitosan samples

In the present study, the shell residue was treated with 0.8 N HCl and 1 M sodium hydroxide for the removal of residual proteins and mineral content because when these concentrations of acid and alkali used the amounts of protein and minerals were reduced to permissible limit for good quality chitin (Shahidi and Synowiecki 1991; No and Meyers 1989; Cira *et al.*, 2002). Characteristics of the chitin prepared were presented in Table 4.10.

Mineral contents of the chitin prepared from the shell residue were given in Fig. 4.9. Here, Ca, Na, K and Mg contents of chitin prepared with lactic acid treated shell residue was found to be less than that of the HCl treated one. This may be because of the high ionization value of lactic acid. Thus, chitin prepared from the lactic acid treated residue was good in quality. Lesser the mineral content, better the quality of chitin (Tolaimate *et al.*, 2003).

FTIR spectra of the chitin prepared from HCl treated fermented residue (Fig. 4.6) and lactic acid treated fermented residue (Fig. 4.7) were compared with that of commercial chitin (Fig. 4.8). The spectra of prepared chitin by fermentation were found to be spectroscopically similar to the FTIR spectra of commercial chitin. The percentage of N-acetylation calculated by the spectra of the prepared chitin was (84.21 and 85.23) also found to be very close to that of commercial chitin (85.86%)

Chitosan was produced from the chitin isolated from fermented residue. Properties of chitosan prepared were given in Table 4.11. Molecular weight and viscosity of chitosan prepared from lactic acid treated samples were found to be

Table 4.10. Characteristics of chitin prepared from the shell residue of *Lactobacillus plantarum* fermentation.

	HCl	Lactic acid
Moisture	6.52±0.25%	6.54±0.41%
Ash	0.76±0.03%	0.52±0.03%
Protein	0.81±0.06%	0.68±0.05%
Chitin	95.41±0.62%	96.52±0.51%
Appearance	White flakes	White flakes
% of N-acetylation	84.21±4.36	85.23±3.18

Table 4.11. Properties of chitosan prepared from hydrochloric acid and lactic acid treated fermented residues of *Lactobacillus plantarum* fermentation

Properties	HCl treated	Lactic acid treated
Viscosity (m Pa.S)	1865±120	2100±149
Molecular weight ($X10^5$) (Da)	2.75±0.16	3.45±0.18
Degree of deacetylaton (%)	95±7	88±6

Fig. 4.5. Protein content in the fermented residue of *Lactobacillus plantarum* after treatment with different alkali concentrations

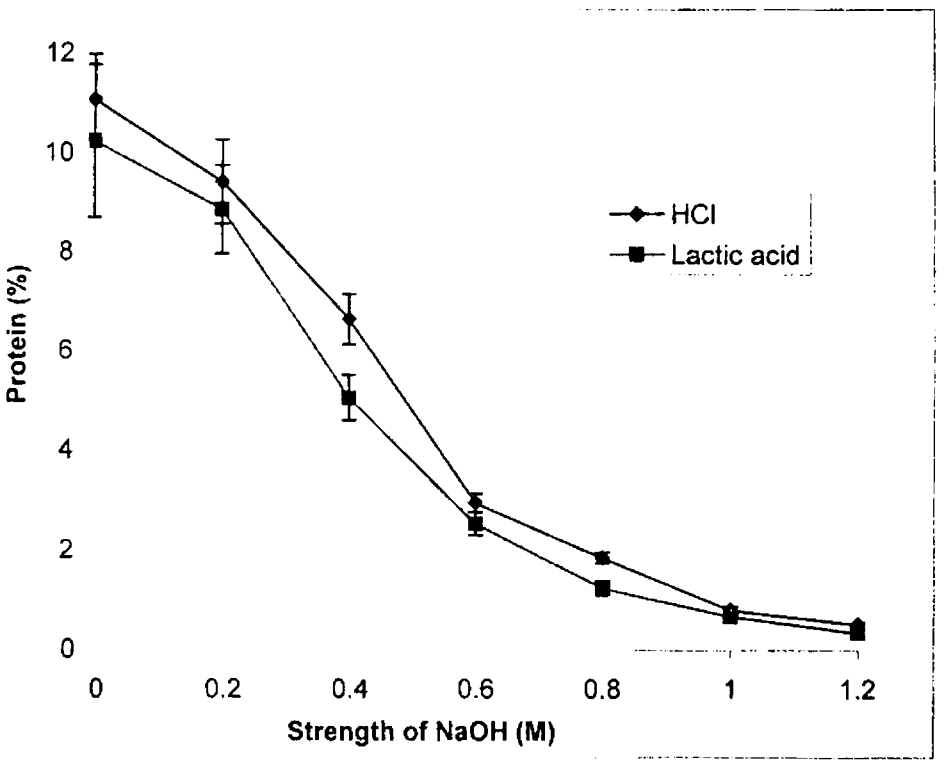


Fig. 4.6. FTIR spectrum of chitin prepared by using *Lactobacillus plantarum*- HCl treated

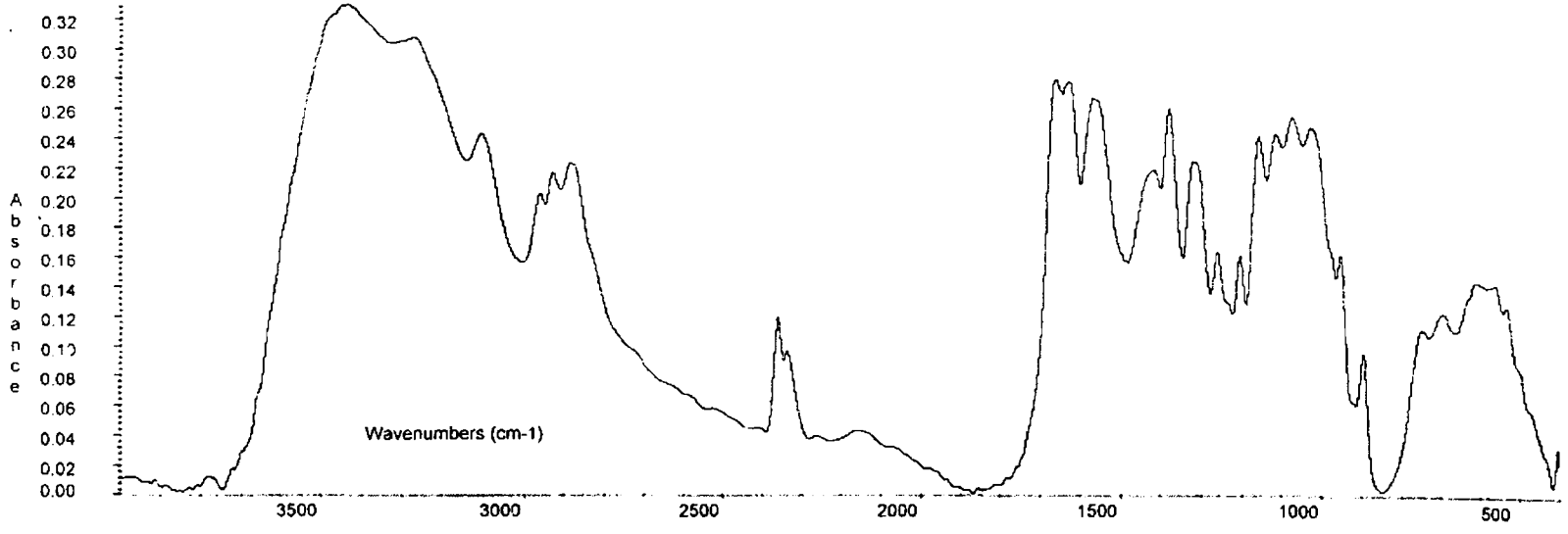


Fig. 4.7. FTIR spectrum of chitin prepared by using *Lactobacillus plantarum*- Lactic acid treated

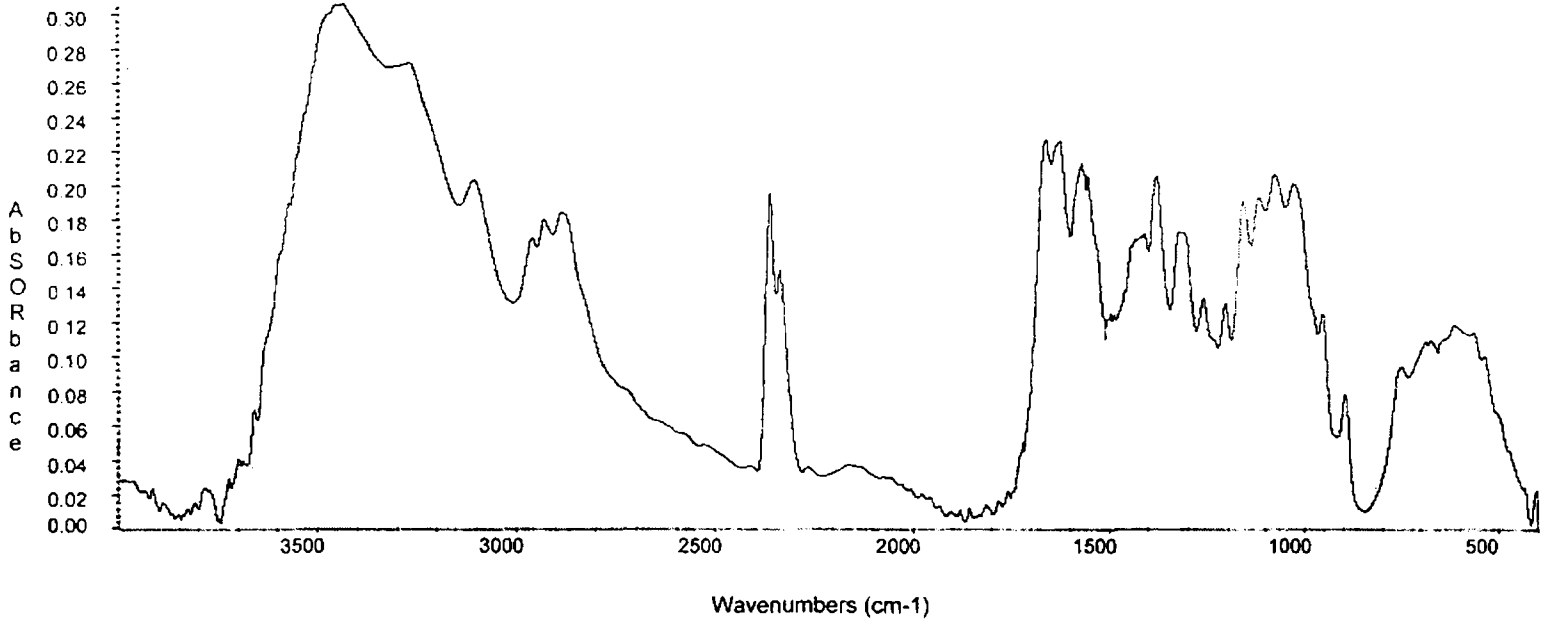


Fig. 4.8. FTIR spectrum of commercially available chitin.

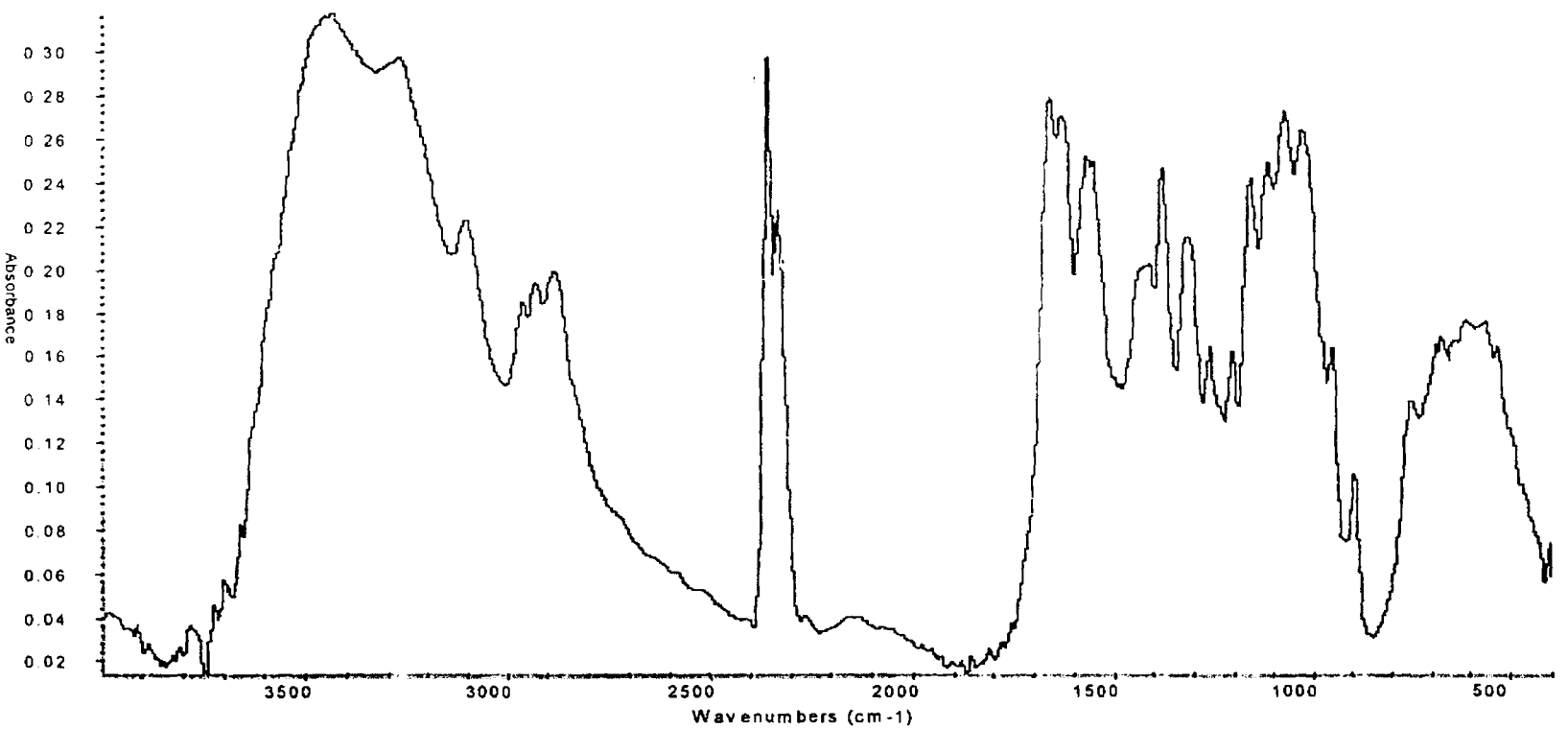
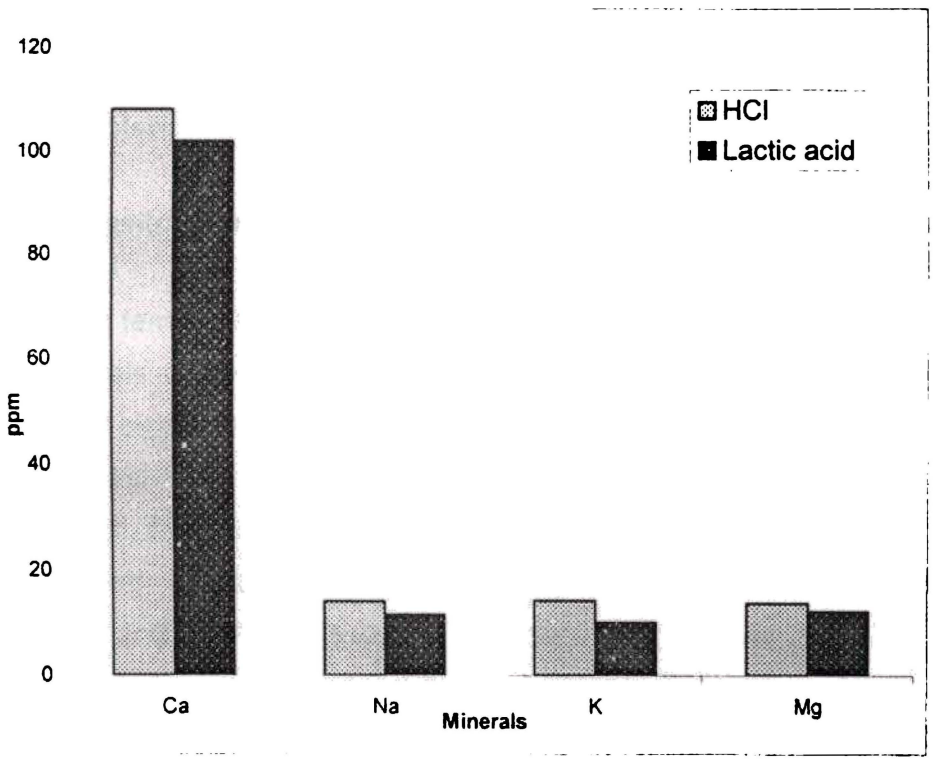


Fig. 4.9. Mineral status of the chitin prepared from the *Lactobacillus plantarum* treated fermented residue



higher than HCl treated ones. Good degree of deacetylation was also noticed in lactic acid treated samples.

Molecular weight and viscosity of chitosan, prepared from the chitin obtained from shell residue, was found to be higher in lactic acid treated samples than HCl treated ones. It can be due to the greater degradation occurred to the HCl treated samples during pH adjustment as it is a strong acid.

4.1.3. Characteristics of protein liquor obtained after fermentation

4.1.3.1. Biogenic amine composition

During fermentation process, an environment of suitable temperatures, pH and high levels of free amino acids over an extended period of time for the production of biogenic amines were created. The presence of biogenic amines in large quantities is an indication of its spoilage which is dependent upon the availability of free amino acids, the presence of decarboxylase positive microorganisms (bacteria containing enzymes which can decarboxylate free amino acids) and conditions favouring bacterial growth (Halasz *et al.*, 1994).

The biogenic composition of the protein liquor obtained after fermentation was studied. Putrescine and spermidine were found to be present in both the HCl treated and lactic acid treated samples. The amino acids, ornitihine and arginine are precursors of putrescine and spermidine is produced from putrescine. (Paterson *et al.*, 1990).

lactic acid treated protein liquor, contains 0.2388 ppm putrescine and 0.3975ppm spermidine. HCl treated sample contains 0.2050 ppm putrescine and 0.3667ppm of spermidine. During lactic acid fermentation, acidic condition produced favours action of biodegradative decarboxylase and acts on amino acid precursor to produce biogenic amines (Tabor and Tabor, 1985).

4.1.3.2. Aminoacid composition

Amino acid composition of the protein liquor obtained from the two treatments were also studied and was presented in the Table 4.12. Protein liquor

Table 4.12. Amino acid composition of the protein liquor (g/100g crude protein) obtained by *Lactobacillus plantarum* treatment of shrimp shell

Amino acids	HCl	Lactic acid
Histidine	4.09	4.34
Lysine	6.21	6.48
Valine	5.25	5.36
Methionine	4.62	4.88
Leucine	6.85	6.93
Isoleucine	4.62	4.85
Phenylalanine	3.22	3.58
Arginine	0.98	0.92
Threonine	4.14	4.25
Tryptophan	0.75	0.74

obtained from fermentation with lactic acid treated shell has found better proportion of essential amino acids as compared to that HCl treated shell. This renders this protein liquor for its incorporation in to cattle feed as a protein supplement. From the results, it was found that there was no deterioration of amino acids by the action of lactic acid. But, in HCl treated samples, the amount of amino acids was a little retarded because of the inorganic acid with high pKa value.

4.1.3.3. Volatile base nitrogen

Total volatile base nitrogen and alpha amino nitrogen of the samples at the end of fermentation were analysed and presented in the Table 4.13.

Both the spoilage parameters were found to be less in lactic acid treated broth than HCl treated broth. This can be due to low pH in lactic acid treated samples than HCl treated ones and which in turn depends on the number of lactic acid bacteria. TVBN and α-amino nitrogen were present in minute quantities and well below than permissible limit. According to Connel (1980), TVBN 100 $mg \cdot 100g^{-1}$ on dry weight basis of salted dried fish could indicate spoilage.

4.2. Fermentation studies with *Lactobacillus brevis*

4.2.1. Optimisation of fermentation parameters

4.2.1.1. Inoculum quantity standardization

0.5 ml of commercial culture of *Lactobacillus brevis* from MRS broth transferred to different concentrations of jaggery solution (10%, 15%, 20%,25% and 30%w/v)produced 10^8CFU/ml after 24 hrs of incubation at 30^0C. So inoculum rate of 10^8 CFU/ml was used for the study.

Lactic acid bacteria are normally present in very low numbers in its biomass and so a suitable starter culture has to be added to the fermenting media to produce sufficient acid and to compete with other flora of the media

Table 4.13. TVBN (Total volatile base nitrogen) and AAN (alpha amino nitrogen) of protein liquor obtained by *Lactobacillus plantarum* treatment of shrimp shell

	HCl	Lactic acid
TVBN (mg%)	37.82±2.36	36.45±2.54
α-amino nitrogen (mg %)	0.56±0.18	0.42±0.17

(Knachel, 1981). According to Weinberg and Muck (1996), minimum inoculum rate of 10^5-10^6cells/gm was required by the lactic acid bacteria to become the dominant flora of the fermenting media. And sufficient acid has to be produced by the added lactic acid bacteria to prevent spoilage and for that also good inoculum rate is necessary. This factor is very important especially in shrimp waste fermentation, as it is a highly perishable commodity.

4.2.2. Optimisation of sugar concentration and fermentation period

4.2.2.1. Changes in pH

The changes in pH was shown in the Table 4.14. In all the treatments, pH showed a first declining trend and thereafter it started increasing. Bacteria utilizes the available sugar and produces acid and low pH was produced during initial days. As fermentation proceeded, available sugar depleted ,so less acid produced and thus pH showed increasing trend at later stages.The changes in available sugar concentration (Table 4.17) and spoilage signs were shown in all the samples when the pH started increasing.

The trend of pH changes was found to be same in all fermentation experiments and it was supported by the work of many authors (Teniola and Odunfa, 2001; Shirai *et al.*, 2001; Cira *et al.*, 2002). The low pH produced at the start of fermentation is due to the acid produced by lactic acid bacteria utilsing the sugar (Rao *et al.*, 2000). Later rise in pH was mainly due to the depletion of sugar for bacterial growth. Calcium solubilised to the media from the shrimp shell by the action of acid produced, acts as a buffer and was also responsible for the rise in pH (Zakaria *et al.*, 1998; Shirai *et al.*, 2001). By the action of proteolytic enzymes in lactic acid bacteria, proteolysis takes place and free amino acids are produced. And the pH rises due to the release of ammonia, when they started utilizing free amino acids as sources of carbon and energy (Shirai *et al.*, 2001). As *Lactobacillus brevis* is hetero fermentative in nature, pH reduction is due to different types of acid produced (Loner and Preve-Akesson, 1988)

4.2.2.2. Changes in microbial count

The changes in microbial count was shown in Table 4.15. From the studies, it was clear that microbial count declined when the available sugar

Table 4.14. Changes in pH on treatment of shrimp shell with *Lactobacillus brevis* *at* differentjaggeryconcentrations with fermentation time

Days	Jaggery concentration				
	10%	15%	20%	25%	30%
0	-	-	-	-	-
2	4.23±0.21	4.21±0.26	4.20±0.15	4.19±0.25	4.16±0.22
4	4.18±0.18	4.13±0.25	4.15±0.16	4.15±0.26	4.13±0.21
6	4.36±0.19	4.28±0.17	4.06±0.21	4.04±0.32	4.01±0.18
8	4.49±0.21	4.38±0.24	4.01±0.22	4.02±0.18	4.00±0.15
10	4.62±0.23	4.53±0.28	4.21±0.14	4.20±0.22	4.11±0.25
12	4.81±0.32	4.61±0.32	4.44±0.36	4.43±0.24	4.45±0.35
14	5.09±0.45	4.86±0.33	4.58±0.34	4.55±0.29	4.53±0.32
16	5.14±0.46	5.20±0.28	4.68±0.34	4.62±0.31	4.68±0.38
18	5.35±0.51	5.38±0.25	4.93±0.28	4.92±0.29	4.97±0.39
20	6.52±0.55	5.49±0.22	5.22±0.26	5.28±0.35	5.23±0.41

substrate depleted. Initially lactic acid bacteria showed a tremendous growth utilizing the carbohydrate source for its energy.

4.2.2.3. Changes in proteolytic activity

The changes in proteolytic activity was given in the Table 4.16. Proteolytic activity first showed an increasing trend in all the treatments and then declined. Deprotenisation from shrimp shell takes place by the proteolytic activity of endogenous and microbial enzymes (Shirai *et al.*, 2001; van Kranenburg *et al.*, 2002). Thus the proteolytic activity is related to number of lactic acid bacteria .In all the treatments it showed maximum activity when maximum concentration of lactic acid bacteria present utilizing the available sugar substrate. According to Dako *et al.* (1995), proteolytic activity decreased in the presence of calcium ions. The decline in the number of microorganisms and the presence of calcium released to the fermenting medium were found to be responsible for the decline in proteolytic activity. In lower concentration jaggery broth, 10% and 15%, it showed maximum activity on 4^{th} and 6^{th} day respectively. In 20%, 25% and 30% fermented broth, it showed maximum activity on the 10^{th} day and after that started decreasing.

4.2.2.4. Changes in sugar concentration

In the present study, jaggery was used as carbohydrate source for the growth of lactic acid bacteria. So the broth of jaggery prepared at different concentrations was used for the inoculum preparation and for fermentation study. Sucrose is the sugar component in jaggery.

Free sugar is an essential substrate for the growth of lactic acid bacteria (Raa *et al.*, 1983) and if shrimp waste alone was added, microbial process will be retarded. So jaggery an easy available carbohydrate source was used for the study. Many authors reported the inoculum preparation using MRS media (Zakaria *et al.*, 1998; Cira *et al.*, 2002) and produced 10 ^{8} CFU/ml in 24 hrs incubation time. But in the present study it was found that it attained 10^{8} CFU/ml in jaggery broth also in 24 h time. Thus by using jaggery as media for inoculum

Table 4.16. Changes in proteolytic activity (g tyrosine/g protein) of fermented samples at differentjaggeryconcentrations with respect to fermentation time

Days	Jaggeryconcentration (%)				
	10	15	20	25	30
0	-	-	-	-	-
2	6.35±0.42	6.58±0.45	6.53±0.42	6.58±0.39	6.58±0.41
4	7.58±0.31	7.64±0.38	7.52±0.58	7.63±0.44	7.63±0.52
6	5.62±0.41	7.85±0.42	9.92±0.65	9.92±0.56	9.92±0.84
8	5.28±0.42	6.42±0.41	10.52±0.81	10.53±0.85	10.56±0.75
10	5.12±0.38	5.62±0.38	10.85±0.89	10.88±0.84	10.88±0.76
12	5.00±0.45	5.42±0.32	9.52±0.53	9.53±0.74	9.55±0.71
14	4.92±0.25	5.38±0.38	8.75±0.51	8.79±0.71	8.79±0.68
16	4.81±0.38	5.02±0.36	6.54±0.48	6.55±0.52	6.52±0.61
18	4.71±0.35	4.92±0.41	6.42±0.44	5.42±0.45	5.42±0.41
20	4.65±0.35	4.74±.32	5.22±0.45	5.24±0.38	5.27±0.45

Table 4.17. The changes in sugar content (mg %) in fermented samples at different jaggery concentrations with respect to fermentation time

Days	Jaggery concentration				
	10%	15%	20%	25%	30%
0	4.68±0.38	6.32±0.54	12.54±0.89	15.54±1.24	18.50±1.17
2	2.90±0.25	4.08±0.38	9.27±0.65	11.09±0.98	13.69±0.97
4	2.03±0.22	3.23±0.25	6.32±0.48	7.65±0.61	9.10 ±0.74
6	1.53±0.14	2.03±0.19	4.27±0.36	5.12±0.42	6.06±0.58
8	1.32 ±0.13	1.85±0.14	3.23±0.27	4.47±0.35	5.26±0.45
10	1.11±0.08	1.13±0.09	2.36±0.21	3.02±0.19	3.49±0.28
12	0.98±0.07	1.01±0.08	2.02±0.21	2.52±0.19	2.99±0.18
14	0.75±0.05	0.97±0.08	1.46±0.19	2.14±0.18	2.02±0.16
16	0.62±0.04	0.82±0.06	1.18±0.08	1.69±0.09	1.72±0.15
18	0.42±0.03	0.62±0.05	1.14±0.09	1.19±0.08	1.43±0.11
20	0.21±0.01	0.43±0.03	0.84±0.07	0.98±0.06	1.18±0.09

development, the cost of the MRS media reduced from the total process cost. During fermentation, lactic acid bacteria utilize sugar and acid was produced as end product (Legaretta *et al.*, 1996). So not only the type of sugar but quantity of sugar is also important for fermentation. According to the amount of sugar available for fermentation, amount of acid was produced. This was clearly evident from the experiment that when sugar substrate depleted, acid production reduced and pH started rising. Many authors reported the utilization of different sources of sugar for fermentation studies (Wang *et al.*, 1997; Zakaria *et al.*, 1998; Rao *et al.*, 2000; Shirai *et al.*, 2001; Evers and Carroll, 1996). According to Cir ı *et al.* (2002), the pH was not reduced below 5.6 if glucose concentration less than 7.5% was used for the fermentation study. So the minimum concentration of jaggery for the study was taken as 10%w/v. In all the five treatments, about 45-55% of sugar was utilized for the inoculum development in the first 24 hours of incubation. And only remaining sugar was available for the fermentation studies. In all the treatments it was found that sugar concentration depleted with fermentation time.

4.2.2.5. Optimisation of sugar concentration

To optimize the sugar concentration, different parameters like pH, total titrable acidity, extent of utilization of sugar, proteolytic activity, percentage of deprotenisation etc were studied. And extent of protein removed from the shell was taken as dependent variable to optimize the sugar concentration. The extent of protein removed from shrimp shell was clearly depicted in the Table 4.18. There was significant difference in the extent of deprotenisation in samples taken from 10%,15% and 20%w/v sugar broths ($P< 0.05$). But in 20%, 25% and 30% broth, there were no significant difference in percentage of deprotenisation. A maximum of 67.5% of protein were removed from shrimp shell at the end of fermentation. It was found that, by increasing the sugar concentration above 10% (amount of jaggery available for fermentation study after the inoculum growth), water activity was reduced due to the large water binding substance, sucrose. Thus the lag phase of lactic acid bacteria extended (Shirai *et al.*, 2001) and its activity reduced. That may be reason for the decreased proteolytic activity of lactic acid bacteria even if sugar concentration increased. So for fermentation

Table 4.18. Percentage of deprotenisation in fermented samples at different jaggery concentrations with respect to fermentation time.

Days	Jaggery Concentration				
	10%	15%	20%	25%	30%
0	-	-	-	-	-
2	13.82±1.12[a]	13.93±1.08[a]	15.22±1.16[a]	15.23±1.36	15.25±1.54
4	21.64±1.82[a]	22.92±1.12[a]	23.52±1.52[a]	23.56±1.45	23.59±1.84
6	36.42±2.10[a]	36.17±2.51[a]	33.64±1.85[a]	33.68±2.14	33.69±2.87
8	43.67±2.51[a]	44.92±3.22[a]	48.91±2.69[a]	48.96±3.84	48.99±2.96
10	43.85±2.56[a]	49.71±3.62[a]	61.25±3.85[a]	61.27±4.21	61.25±3.56
12	48.89±3.21[a]	56.86±4.15[a]	62.83±3.95[a]	64.87±4.12	64.89±3.87
14	59.96±3.25[a]	61.89±4.21[a]	63.32±4.26[a]	64.33±4.52	65.33±3.96
16	61.01±4.25[a]	65.92±3.65[a]	66.33±4.51[a]	67.33±4.86	67.34±3.84
18	61.26±3.87[a]	67.11±3.97[a]	67.34±4.56[a]	67.34±5.64	67.35±4.65
20	62.35±3.96[a]	67.26±3.98[a]	67.34±4.84[a]	67.35±5.26	67.35±4.21

[a] $P<0.05$ significantly different compared with different jaggery concentrations

process using *Lactobacillus brevis* for the production of chitin, 20%w/v jaggery broth was selected for the study.

4.2.2.6. Optimisation of fermentation time

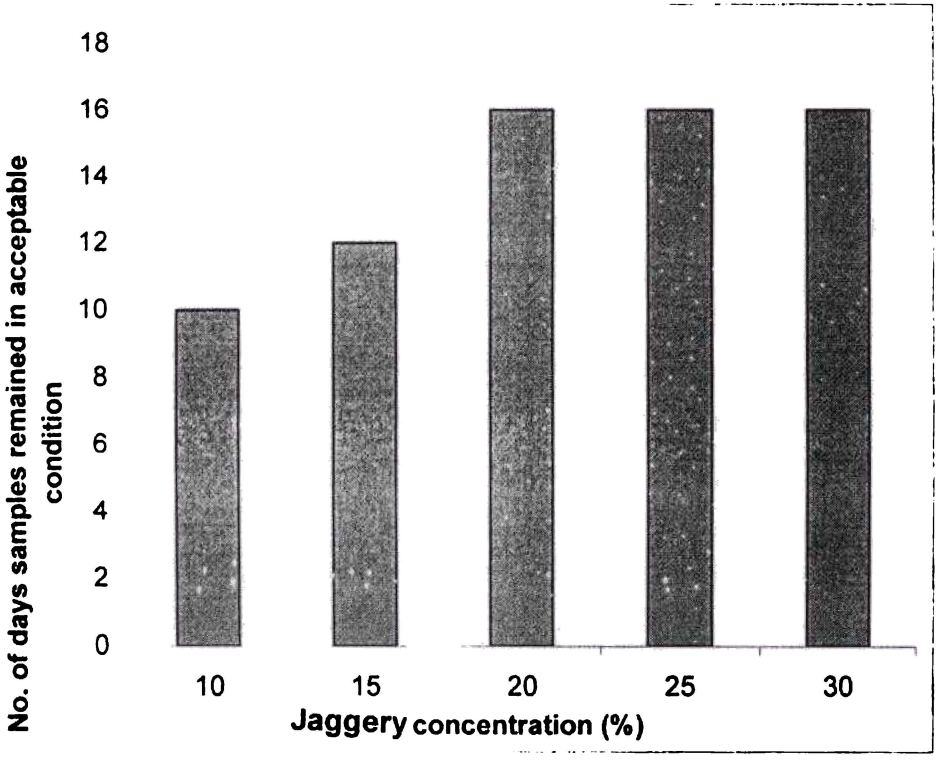
To optimize the fermentation period, spoilage of the sample and percentage of deprotenisation were considered. The number of days the samples in different sugar broths remained in acceptable condition was shown in the Fig. 4.10.

At 10% jaggery broth, signs of spoilage like off odour, presence of mould on the surface etc was observed on the 8^{th} day, well in correlation with pH rise and sugar depletion and it remained in acceptable condition till 10^{th} day according to odour evaluation done by the panelists. Acceptable conditions were observed in 15% jaggery broth on 12^{th} day and 20%, 25% and 30% jaggery broth on 16^{th} day.

During fermentation, the low pH and different antimicrobial compounds released by lactic acid bacteria like H_2O_2, acetoin and bacteriocins prevents spoilage bacteria (Zakaria *et al.*, 1998; Raa *et al.*, 1983; Hugas and Monfort, 1997). But as fermentation proceeds, due to the proteolytic activity of lactic acid bacteria, proteins are break down to small peptides and this reduced the water activity during fermentation (Shirai, 1999, Horner, 1997). Thus the reduction in water activity affects the growth of lactic acid bacteria and spoilage bacteria started their activity. That may be the reason for signs of spoilage found in all the treatments. And in higher sugar concentrated broth, water activity reduced due to the presence of water binding sugar, and the lag phase of lactic acid bacteria extended (Shirai *et al.*, 2001). And spoilage bacteria compete with lactic acid bacteria for the metabolizable sugar and putrefaction occurs.

And there were no significant difference in the extent of deprotenisation on 16^{th}, 18^{th} and 20^{th} day. So by considering the percentage of deprotenisation and spoilage factor, 16 days of incubation was taken for the study.

Fig. 4.10. Number of days the samples remained in acceptable condition during fermentation study at different jaggery concentrations.



4.2.3. Fermentation of shrimp shell using *Lactobacillus brevis* for the production of chitin and chitosan

4.2.3.1. A comparitive study -The initial pH of the shrimp shell adjusted with an organic acid (Lactic acid) and inorganic acid (HCl)

4.2.3.1.1. Changes in pH

The changes in pH was given in the Table 4.19. The changes in pH was found to be similar to that observed in fermentation using *Lactobacillus plantarum*. *Lactobacillus brevis* is a hetero fermentative in nature,and so pH reduction is not only due to lactic acid (Teniola and Odunfa, 2001).

Fermentative bacteria first utilizes the metabolisable sugar available in the substrate for its energy and carbon source.It converts the sugar to acidic end products via pyruvate to produce energy (Leroy and de Vuyst, 2003). Thus the pH decreased in accordance with the acid production. Later when the sugar source depleted, it started utilizing the proteinaceous matter in the shell and produced small peptides and aminoacids. Ammonia is produced by the utilization of aminoacids by the microorganism (Allaghẽny *et al.*, 1996). Thus the pH started rising. Acid produced caused the deminerlisation of shrimp shell and calcium solubilised from the shell also responsible for the hike in pH (Shirai *et al.*, 2001).

The pH reduction and the quantity of acid produced was found to be more in the lactic acid treated samples than HCl treated samples. This difference may be due to the destruction of quantity of starter culture by HCl when it was utilized for initial pH adjustment.

4.2.3.1.2. Changes in microbial count and sugar concentration

The changes in microbial count was shown in the Table 4.20. 0.5 ml of inoculum inoculated into the jaggery broth produced 10^8 CFU/ml and it utilizes about 45% of jaggery in the first 24 hrs of incubation (Fig. 4.11).The microbial count increased in both HCl and lactic acid treated samples utilizing the sugar source and when the sugar source depleted ,a general decrease in count was

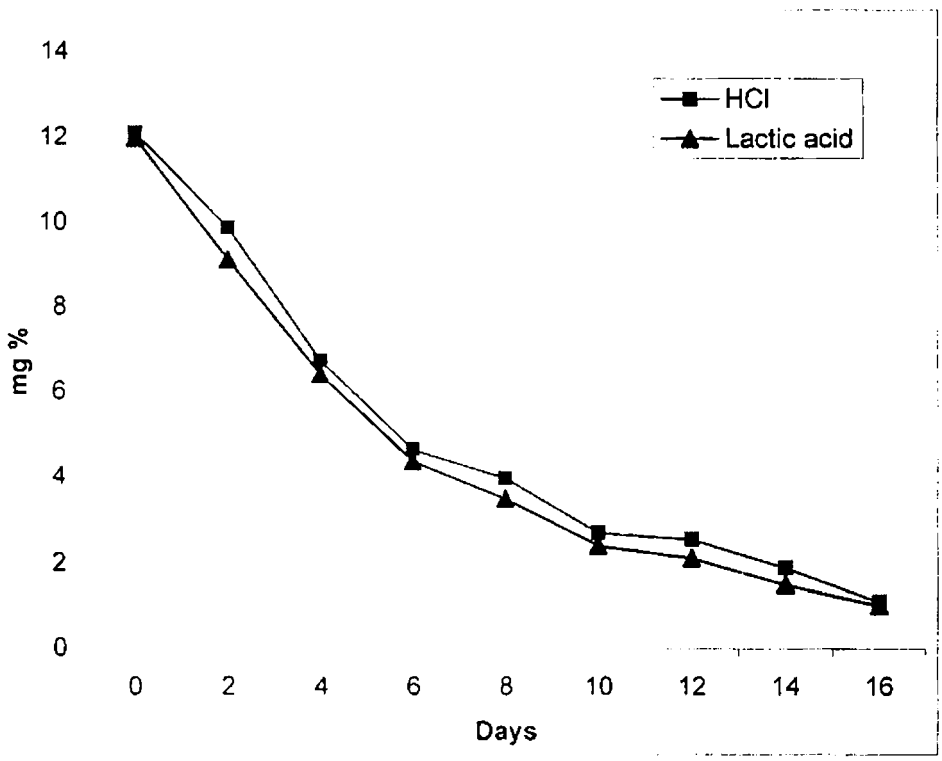
Table 4.19. Changes in pH during fermentation samples treated with *Lactobacillus brevis* where the initial pH was adjusted with HCl and lactic acid.

DAYS	HCl	Lactic acid
0	-	-
2	4.36±0.26	4.24±0.31
4	4.47±0.22	4.18±0.34
6	4.18±0.36	4.1±0.26
8	4.06±0.31	4.0±0.21
10	4.04±0.27	4.01±0.28
12	4.14±0.24	4.11±0.31
14	4.62±0.35	4.48±0.36
16	4.79±0.39	4.67±0.39

Table 4.20. Changes in bacterial count (CFU/ml) of the ensilage produced by the fermentation with Lactobacillus brevis

Days	HCl	Lactic acid
0	$2.13X10^8$	$2.20X10^8$
2	$2.20X10^9$	$2.43X10^9$
4	$2.46X10^9$	$2.58\ X10^9$
6	$2.73X10^9$	$2.81X10^9$
8	$2.89X10^9$	$2.96X10^9$
10	$2.92X10^9$	$2.97X10^9$
12	$2.56\ X10^8$	$2.65X10^8$
14	$2.14\ X10^8$	$2.38X10^8$
16	$1.98\ X10^8$	$1.99\ X10^8$

Fig 4.11. Changes in jaggery concentration of fermenting samples during ensilation with *Lactobacillus brevis*



noticed. The extent of utilization of sugar was given in Fig. 4.11. At the end of fermentation about 95% of sugar was utilised by the lactic acid bacteria. When the sugar source depleted, it depends on protein matter for its energy. Spoilage and off odour was noticed in both the samples when they started utilizing the amino acids and ammonia was released (Shirai *et al.*, 2001).

The number of microbial organisms was found to be more in lactic acid treated samples than HCl treated samples. Accordingly the utilization of sugar was found to be more in lactic acid treated samples.

4.2.3.1.3. Deprotenisation and demineralisation

The extent of deprotenisation with fermentation time was given in the Table 4.22. The proteins are removed from the shell with the help of proteolytic enzymes present in lactic acid bacteria (Shirai *et al.*, 2001). The trend of activity of proteolytic enzymes was shown in the Table 4.21. It showed an increasing trend upto 10^{th} day and thereafter it started decreasing. This has good correlation with the number of lactic acid bacteria (Table 4.20). When the count started reducing with sugar depletion, its activity also declined. And the calcium ions solubilised from shrimp shell due to the action of acid produced also inversely affected the proteolytic activity (Dako *et al.*, 1995). About 67.5% and 68.1% of proteins were removed from the shrimp shell treated with HCl and lactic acid respectively.

In lactic acid treated samples, deprotenisation was found to be more than HCl treated samples. This was due to the fact that proteolytic activity is directly proportional to the number of microorganisms.

The acid used for the initial pH adjustment affected the percentage of demineralization. Its trend was shown in the Table 4.22. In the initial days, HCl treated samples showed greater demineralization than lactic acid treated samples. This difference may be due to the difference in strength of the two acids. In the following days, demineralization was brought by the acid produced by the lactic acid bacteria. At the end of fermentation, 66.3% and 67.4% of ash

Table 4.21. Changes in proteolytic activity (g tyrosine/ g protein) of *Lactobacillus brevis* during fermentation.

Days	HCl	Lactic acid
0	-	-
2	6.2111±0.34	6.7241±0.36
4	7.2619±0.42	7.6892±0.41
6	9.2521±0.54	9.6584±0.56
8	10.0143±0.43	10.6347±0.51
10	10.4589±0.42	10.8756±0.49
12	9.3596±0.52	9.7216±0.48
14	8.3452±0.38	8.9689±0.42
16	6.5296±0.39	6.8875±0.42

Table 4.22. Percentage of protein and ash removed from the shell during fermentation with *Lactobacillus brevis*

Days	HCl treated		Lactic acid treated	
	%of protein removed	%of ash removed	%of protein removed	%of ash removed
0	-	15.62±0.68	-	7.92±0.62
2	15.92±0.81	22.85±1.78	16.62±1.34	16.65±1.38
4	24.63±1.26	28.22±1.65	25.33±1.76	23.53±1.76
6	30.74±1.85	34.54±2.32	32.62±2.33	29.84±3.54
8	38.51±3.46	40.24±3.65	40.48±3.48	36.91±3.13
10	47.35±3.66	48.92±4.23	48.62±4.12	49.25±4.58
12	54.32±4.11	56.44±4.58	56.64±5.42	56.96±4.14
14	60.91±5.22	61.65±5.26	62.26±5.44	62.04±5.47
16	67.52±3.26	66.32±4.29	68.15±4.83	67.48±4.12

was removed from HCl treated and lactic acid treated samples respectively. In the following days of fermentation, in HCl treated samples acid production was less and that was reflected in the extent of demineralization. It may be due to the destruction of inoculum quantity when HCl was used for initial pH adjustment.

4.2.3.2. Mild chemical treatments of fermented residue to produce chitin

Table 4.23 compares protein and ash contents of the fermented residue with that of the raw material

Fig. 4.12 shows the percentage of protein content in the fermented residue treated with different concentrations of sodium hydroxide.

Different concentrations of sodium hydroxide treated with fermented residue removed the residual protein and it was found that 1.2 M and 1.4M NaOH treatments were most efficient in reducing the residual protein level less than 1%. So we have chosen less strength 1.2 M NaOH for the production of chitin.

Fig. 4.13. shows the percentage of ash content in the fermented residue treated with different concentrations of hydrochloric acid. Among the different concentrations of acid treatment to remove the residual ash content, 1N, 1.2 N and 1.4N HCl treatments reduced the ash content to less than 1% in the fermented residue. So, the minimum concentrated HCl (1N) was chosen for the study.

Chitosan was prepared from the chitin.

4.2.3.3. Properties of chitin and chitosan prepared

The properties of chitin prepared were presented in the Table 4.24. Chitin prepared from HCl treated and lactic acid treated fermented residue showed almost similar properties.

Mineral content of the chitin samples were presented in the Fig. 4.14. Calcium content was found to be more in both the samples. This was due to the

Table 4.23. Comparison of protein and ash content of the *Lactobacillus brevis* fermented residue with the raw material

Particulars	% of protein content	% of ash content
Raw material	35.96±3.15	19.25±1.12
HCl treated	11.68±1.16	6.49±0.15
Lactic acid treated	11.47±1.01	6.27±0.26

Table 4.24. Properties of chitin prepared from the shell residue obtained by *Lactobacillus brevis* fermentation

	HCl	Lactic acid
Moisture	6.81±0.3%	6.84±0.4%
Ash	0.751±0.04%	0.729±0.03%
Protein	0.815±0.05%	0.802±0.04%
Chitin	94.6±0.6%	94.9±0.5%
Appearance	White flakes	White flakes
% of N-acetylation	84.4±5.3	85.6±5.1

Fig. 4.12. Percentage of protein content in fermented residue subjected to different NaOH concentrations

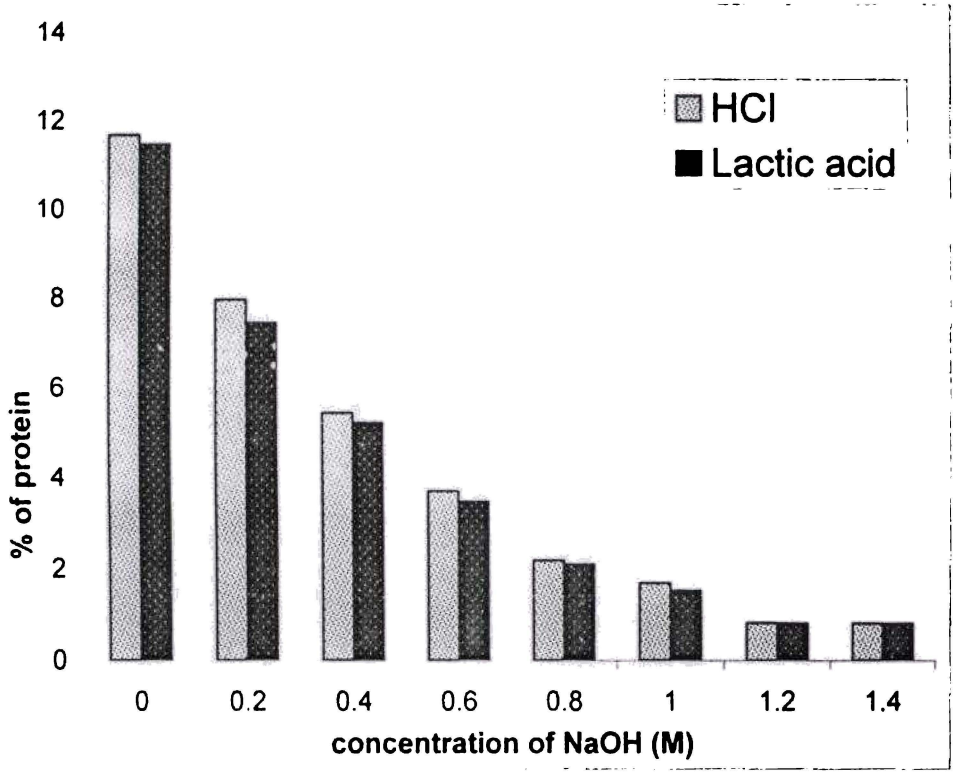


Fig. **4.13.** Percentage of ash content in the fermented residue subjected to different HCl concentrations.

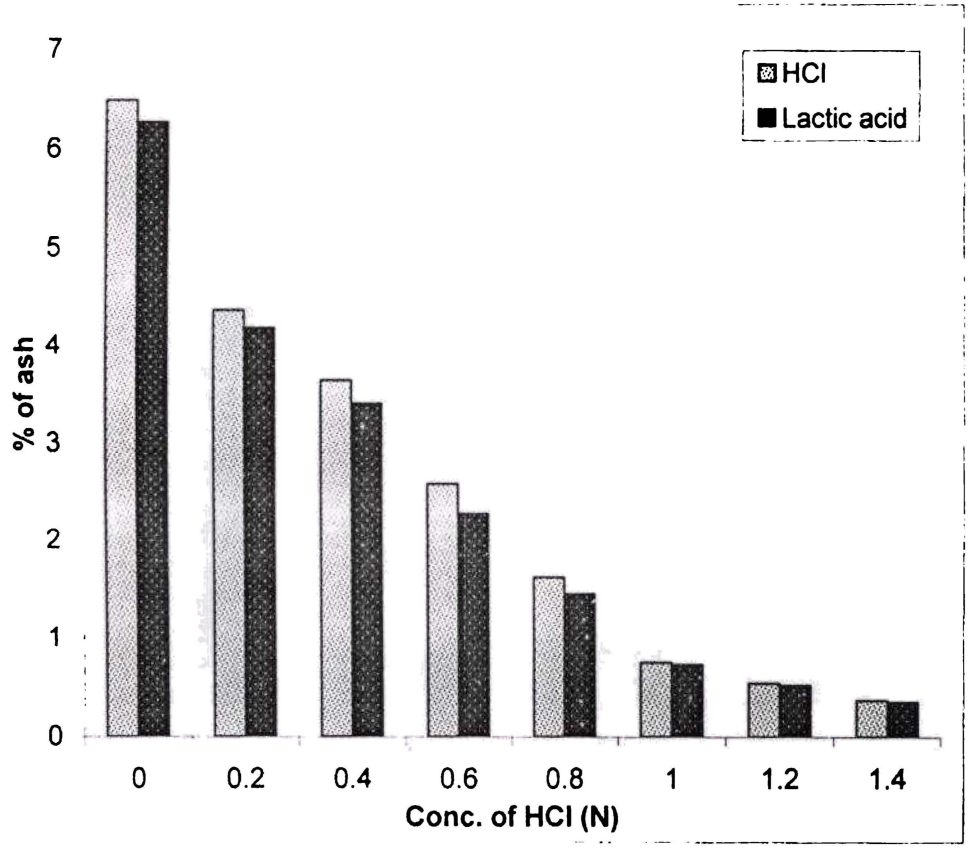
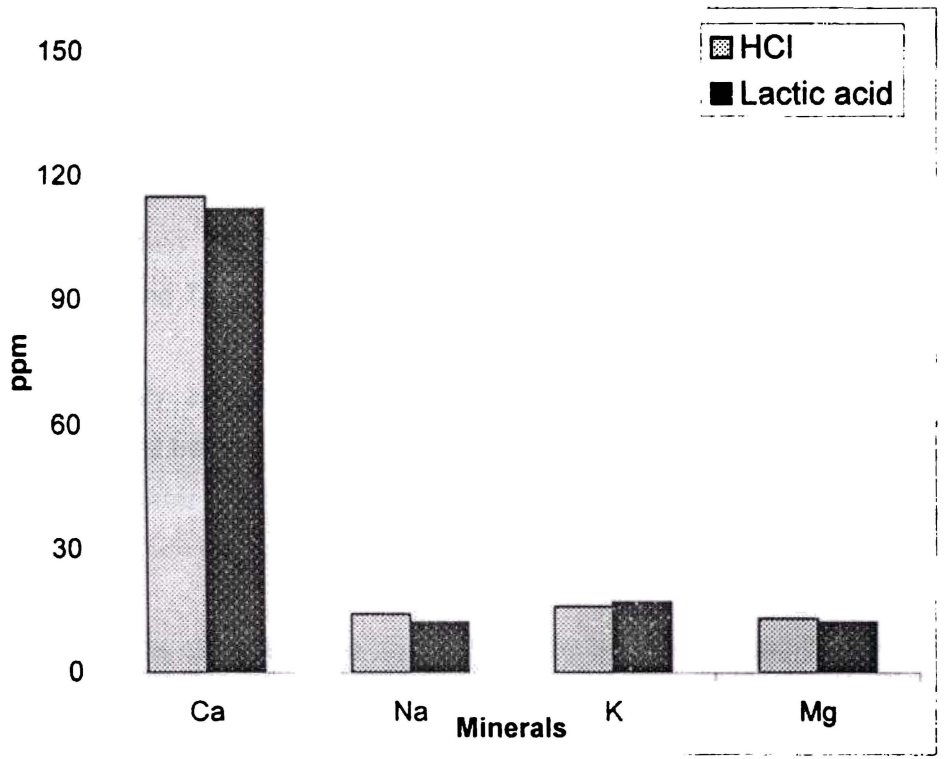


Fig. 4.14. Mineral content of chitin prepared from the fermented residue obtained after the treatment with *Lactobacillus brevis*



fact that the main constituent of shrimp shell is calcium carbonate (Legarreta *et al.*, 1996).

FTIR spectra of chitin prepared from *Lactobacillus brevis* fermented residue were shown in Figs 4.15 and 4.16. There was not much difference found in the spectra of HCl and lactic acid treated chitin samples. Percentage of N-acetylation values calculated from the spectra were 84.4 and 85.6 for HCl and lactic acid treated samples respectively. These values were marginally different

Properties of chitosan prepared from the two chitin samples were presented in the Table 4.25.

Viscosity and molecular weight were found more in chitosan prepared from lactic acid treated samples than HCl treated samples (Table 4.25). This can be due to greater degradation that happened in HCl treated samples during pH adjustment.

From the studies it was found that lactic acid was better than HCl for initial pH adjustment.

4.2.4. Characteristics of protein liquor obtained after fermentation

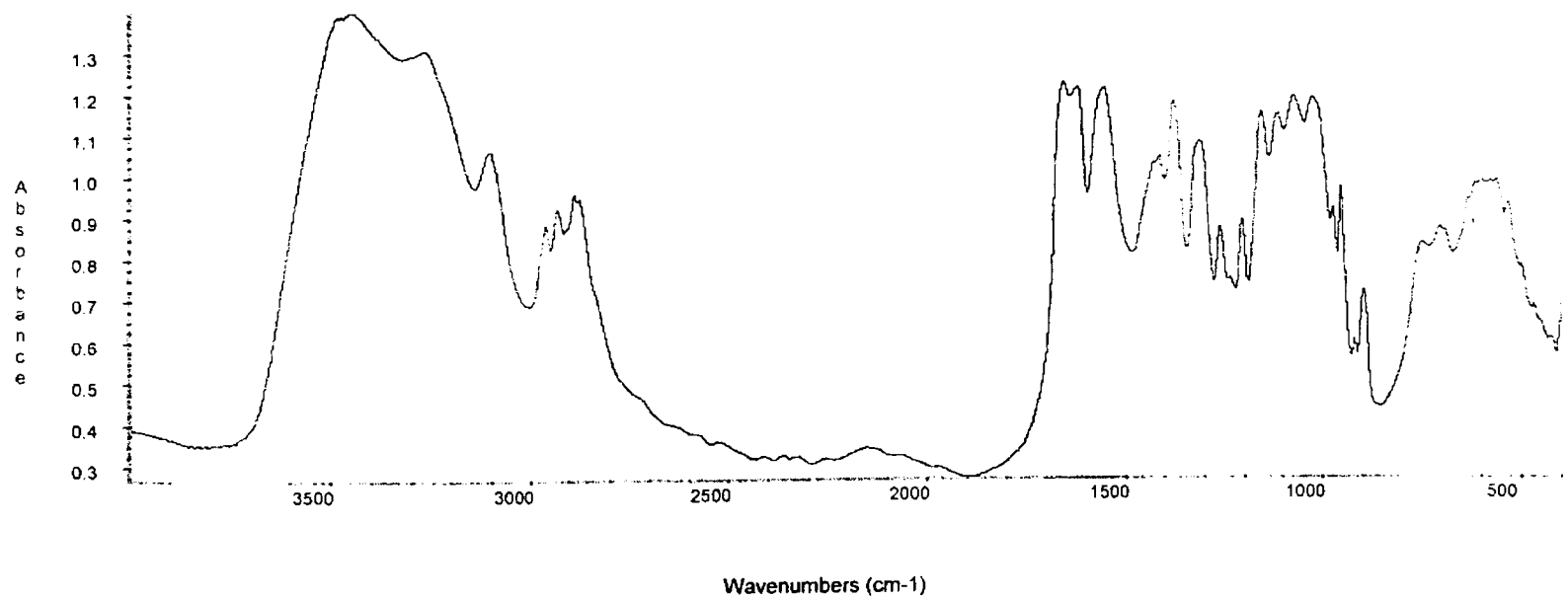
4.2.4.1. Biogenic amine composition

The biogenic amine composition of the protein liquor obtained after fermentation was studied. It was found that putrescine was present in both the samples in minute quantities. Lactic acid treated broth, contained 0.1655 ppm and HCl treated broth contained 0.1357 ppm putrescine. Putrescine, the biosynthetic precursor of polyamines, was produced by two pathways. One by the decarboxylation of ornithine and another by the decarboxylation of arginine to agmatine (Morris and Jorstad, 1970; Moreno-Arribas *et al.*, 2003). In shrimp waste ensilage, biodegradative decarboxylase were produced, when cells were

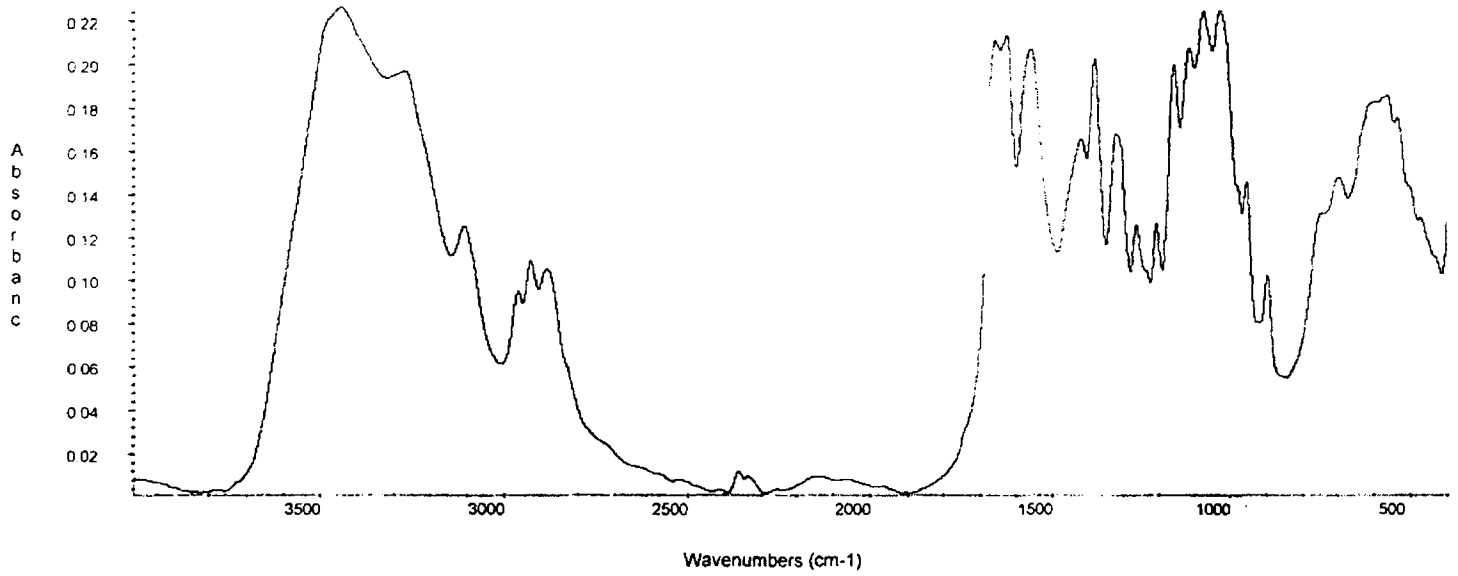
Table 4. 25. Properties of chitosan prepared with the chitin obtained from *Lactobacillus brevis* treated fermented residue.

Properties	HCl	Lactic acid
Moisture (%)	7.7±0.5	7.8±0.3
Appearance	Off white flakes	Off white flakes
Acid Insoluble ash	Nil	Nil
Solubility (10%) in 1%acetic acid	95%	93%
Viscosity (m Pa.S)	1680±138	1742±124
Molecular weight ($X10^5$) (Da)	2.56±0.15	2.81±0.12
Degree of deacetylaton (%)	84±6	81±7

4.15. FTIR spectrum of chitin prepared by using *Lactobacillus brevis*- HCl treated



4.16. FTIR spectrum of chitin prepared by using *Lactobacillus brevis*- Lactic acid treated



grown in an acidic enriched medium containing the corresponding amino acid precursor produced by the break down of proteins (Tabor and Tabor, 1985).

4.2.4.2. Amino acid composition

Amino acid composition of the protein liquor obtained from the two treatments were also studied and was presented in the Table 4.26. Here also , it is well clear that the protein liquor obtained from lactic acid treated shell reside, has more quantity of amino acid than in HCl treated one.

This may be because of the less dissociation property of lactic acid.

4.2.4.3. Volatile base nitrogen

Total volatile base nitrogen and alpha amino nitrogen of the samples at the end of fermentation were analysed and presented in the Table 4.27.

Both the spoilage parameters were found to be less in lactic acid treated broth than HCl treated broth. This can be due to low pH in lactic acid treated samples than HCl treated ones and which in turn depends on the number of lactic acid bacteria.

4.3. Fermentation experiments using *Bacillus subtilis*

4.3.1. Optimisation of fermentation parameters

4.3.1.1. Type and Quantity of inoculum

Bacillus subtilis neutral protease was commonly used as the commercial starting material for fermentation (Lauer *et al.*, 2000). Besides neutral protease, it has amylases also to act on carbohydrates. Calcium is required for the structural stability of the protease enzyme. And amylases of Bacillus species are believed to be metallo enzymes having calcium as the cofactor (Fisher and Stein, 1960). When combined with calcium ions, these amylases are resistant to proteolysis (Fisher *et al.*, 1958) and also it prevents neutral protease autolysis (Tsuru *et al.*, 1964). So for the present study *Bacillus subtilis* was selected because of its

Table 4.26. Amino acid composition (g/100g crude protein) of the protein liquor obtained by *Lactobacillus brevis* fermentation

Amino acids	HCl	Lactic acid
Histidine	4.21	4.72
Lysine	6.01	6.42
Valine	5.32	5.49
Methionine	4.68	4.81
Leucine	7.62	6.86
Isoleucine	4.53	4.62
Phenylalanine	3.86	3.98
Arginine	1.14	1.23
Threonine	4.25	4.37
Tryptophan	0.64	0.69

Table 4.27. Total volatile base nitrogen (TVBN) and α-amino nitrogen contents of the protein liquor obtained after fermentation with *Lactobacillus brevis*

	HCl	Lactic acid
TVBN (mg%)	43.42±2.15	28.08±1.42
α-amino nitrogen(mg %)	0.87±0.12	0.68±0.15

proteolytic nature and calcium is present in the substrate for its stability. And also optimum activity of Bacillus is around neutral pH and shrimp shell was found to be good substrate for its activity. Because of its amylase activity, it produces acid by its action on the sugar source provided for its growth and thus prevents the action of spoilage bacteria.

0.5 ml of commercial culture of *Bacillus subtilis* from nutrient broth transferred to different concentrations of jaggery solution (10%, 15%,20%,25% and 30%w/v)produced 10^8CFU/ml after 24 h of incubation at 30^0 C. So inoculum rate of 10^8 CFU/ml was used for the study. The growth of bacillus species was rapid during the first 18-24h time and it produces more than 10^8 CFU/ml of cells (Kiers *et al.*, 2000).

4.3.1.2. Optimisation of sugar concentration

Based on the extent of demineralization and deprotenisation, sugar concentration for the present study was standardized.

The extent of protein and ash removed from the shell were shown in the Tables 4.28 and 4.29. The neutral protease in *Bacillus subtilis* was responsible for the proteolysis of the shrimp shell (Bustos and Healy, 1994; Lauer *et al.*, 2000). It was found that their activity was more in the first 48hrs of fermentation, when they attained a pH around 6. According to Yang *et al.* (2000), they require an optimum pH of 6 and optimum temperature of 30^0 C for their maximum proteolytic activity. There was significant difference in the extent of deprotenisation between samples from 10%, 15% and 20% sugar broth ($P<0.05$).But there was no significant difference in the percentage of protein removed in the samples taken from 20%, 25% and 30%. A maximum of 84 % of protein was removed from the shrimp shell at the end of fermentation. Demineralisation also occurred to the shrimp shell due to the acid produced by the Bacillis species. Sarkar *et al.* (1993) earlier reported about their acid production potential utilizing sugars as substrates for their growth. Acid produced reacts with calcium carbonate present in the shrimp shell and solubilised as its salt. This demineralization percentage was also higher in the initial fermentation

Table 4.28. Percentage of deprotenisation of fermented samples at different jaggery concentrations with respect to fermentation time.

Days	jaggery concentration				
	10	15	20	25	30
0	-	-	-	-	-
2	32.51 ±2.61[a]	32.47±2.45[a]	33.42±1.89[a]	34.25±2.41	34.96±2.14
4	38.42±2.45[a]	39.02±2.20[a]	41.64±2.69[a]	41.98±2.36	42.26±2.58
6	50.25±3.85[a]	51.45±2.96[a]	53.89±3.12[a]	54.21±3.56	54.69±3.47
8	53.59±2.96[a]	62.00±3.85[a]	64.10±4.09[a]	64.56±3.95	64.98±4.56
10	57.89±3.54[a]	69.23±3.69[a]	72.85±3.25[a]	73.24±4.51	73.59±4.98
12	60.08±4.31[a]	72.85±3.48[a]	79.56±3.48[a]	79.97±4.15	80.09±5.87
14	63.33±4.08[a]	74.94±4.36[a]	84.31±3.96[a]	84.33±5.84	84.33±5.69
16	65.56±4.69[a]	75.21±4.85[a]	84.33±4.81[a]	84.33±5.45	84.36±6.28
18	66.05±5.48[a]	75.54±4.96[a]	84.34±4.45[a]	84.35±5.84	84.36±6.48
20	66.25±5.29[a]	76.26±5.28[a]	84.34±4.69[a]	84.36±5.62	84.37±6.96

[a] $P<0.05$ significantly different compared with different jaggery concentrations

Table 4.29. Percentage of demineralisation of fermented samples at different jaggery concentrations with respect to fermentation time

Days	jaggery concentration				
	10	15	20	25	30
0	15.42±1.52[a]	15.87±1.94[a]	16.15±1.92[a]	16.85±1.87	16.83±1.94
2	34.52±1.85[a]	34.98±2.54[a]	35.61±2.41[a]	35.9±1.96	36.01±2.65
4	44.87±2.15[a]	44.92±2.58[a]	46.23±2.56[a]	46.87±3.14	46.98±2.54
6	50.25±3.85[a]	53.82±2.96[a]	56.84±2.85[a]	56.93±4.52	56.99±2.87
8	52.47±3.96[a]	54.69±2.98[a]	63.78±2.74[a]	63.82±4.63	63.97±3.68
10	52.68±3.48[a]	55.94±3.09[a]	67.25±3.56[a]	67.82±4.68	68.12±3.69
12	52.72±3.95[a]	56.05±3.85[a]	70.69±4.18[a]	70.82±5.85	71.05±4.18
14	52.84±3.87[a]	56.24±3.95[a]	72.56±4.19[a]	72.63±5.46	72.95±4.29
16	52.96±4.21[a]	56.43±3.45[a]	72.69±5.47[a]	72.70±5.63	72.95±4.58
18	53.12±4.20[a]	56.68±4.52[a]	72.85±5.85[a]	72.86±6.25	72.96±4.96
20	53.34±3.98[a]	56.81±4.69[a]	72.86±5.63[a]	72.87±6.85	72.97±4.98

[a] $P<0.05$ significantly different compared with differentjaggeryconcentrations

days due to the greater acid production. Subsequently when the sugar concentration in the fermenting broth depleted, acid production also declined. In the experiments it was found that there were significant difference in the extent of demineralization in samples taken from 10%, 15% and 20% sugar broth ($P<0.05$). But significant difference in the percentage of demineralization was found in the samples taken from 20%, 25% and 30% sugar broths. About 72% of minerals were removed from the shrimp shell at the end of fermentation.

In higher concentrated sugar broths, water activity was greatly affected by the metabolisable sugar and thus it reduced the microbial count. This was clearly reflected in the extent of deprotenisation and demineralisation. So, low concentrated sugar broth 20% w/v was selected for the study.

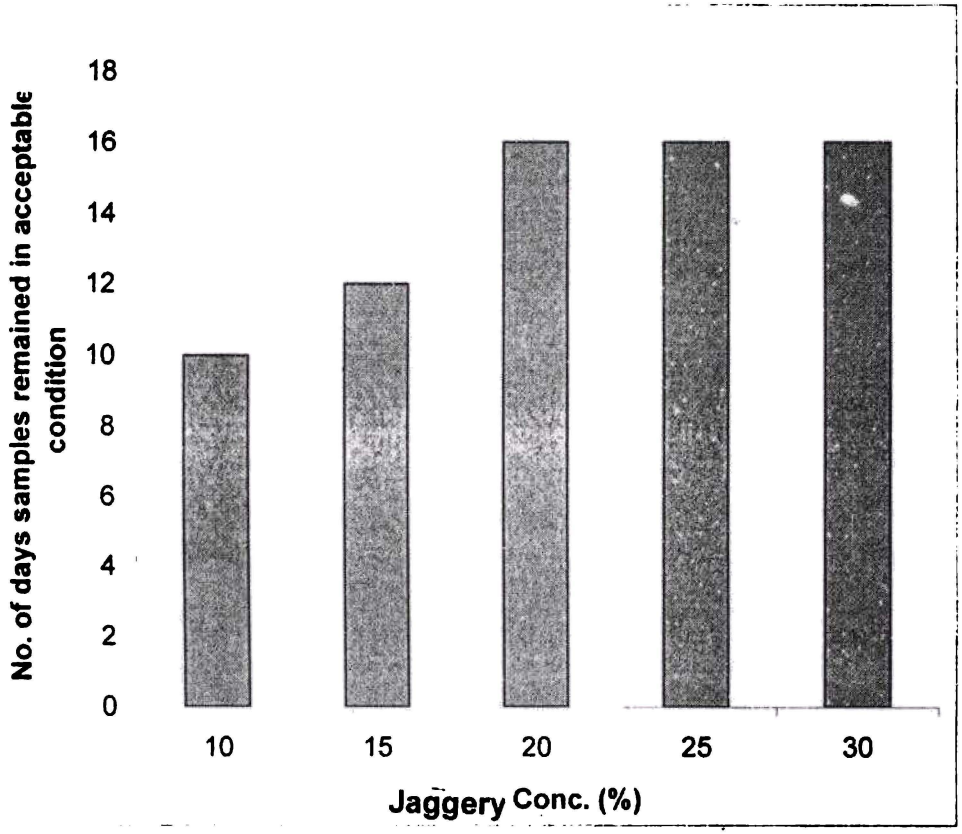
4.3.1.3. Optimisation of Fermentation Period

For optimizing the fermentation period, odour evaluation results, pH changes, extent of demineralization and deprotenisation wee studied.

Based on the odour evaluation done by the panelists, spoilage of sample in different sugar broths was studied. The number of days the sample remained in acceptable condition was represented in the Fig. 4.17. Spoilage of the sample was indicated by the off odour and presence of mould on the surface of the broth. With fermentation time, due to the break down of proteins to amino acids, water activity of the media was reduced and thus spoilage bacteria started their activity. The rise in pH also favours their activity.

In 10% sugar broth showed spoilage signs on 6^{th} day and it remained in acceptable condition till 10^{th} day. Similarly 15% remained in acceptable condition till 12^{th} day and 20%, 25% and 30% remained for 16 days. In low concentrated sugar broths (10% and 15%), when the available sugar source depleted it started using the proteins and showed early spoilage signs of putrefaction. And in high concentrated sugar broth (20%, 25% and 30%), showed spoilage signs on the same day. Reduced water activity due to high sugar content affected the microbial count. Studies found that there was no significant difference in the

Fig. 4.17. The number of days samples remained in acceptable condition during fermentation with *Bacillus subtilis* at different jaggery concentration.



extent of deprotenisation and demineralisation in the samples taken on 14th, 16th, 18th and 20th day. But significant difference was found between 2nd, 4th, 6th, 8th, 10th, 12th and 14th day (P<0.05).

Thus considering the spoilage and degree of deprotenisation and demineralisation, 15 days of fermentation study using 20% w/v sugar broth was selected.

4.3.2. Fermentation of shrimp shell using *Bacillus subtilis* for the production of chitin and chitosan (Sini *et al.*, 2007)

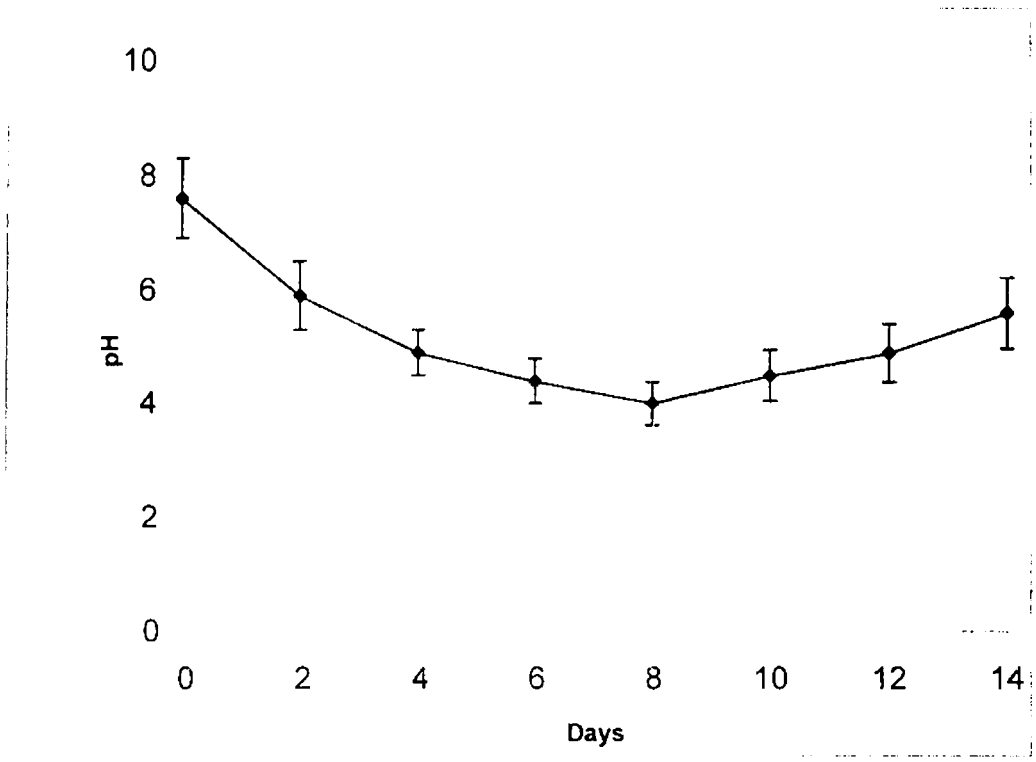
4.3.2.1. Changes in pH

During fermentation, pH of the liquor samples first showed decreasing trend upto 8th day, thereafter it started rising (Fig. 4.18). An initial decrease in pH was noticed in samples and that could be due to the ability of the strain *Bacillus subtilis* to initially use sugars as substrates for their growth (Sarkar *et al.*, 1993) and simultaneously they produce acid via pyruvate (Leroy and de Vuyst, 2003). Low pH condition prevents the decay of shrimp shell, which are colonized by the spoilage and pathogenic organisms (Zakaria *et al.*, 1998). By 8th day, about 87% of sugar in the jaggery broth was utilized by the organism. So, when the sugar substrate was depleted, acid production decreased and pH started increasing (Legarreta *et al.*, 1996). The acid produced was responsible for the extent of demineralization noticed in the samples at the end of fermentation (Rao *et al.*, 2000). During fermentation, proteolysis occurs and ammonia is released due to utilization of amino acids for the growth of the bacteria (Sarkar *et al.*, 1993; Kiers *et al.*, 2000; Ohta, 1986; Steinkraus, 1992; Allagheny *et al.*, 1996).This also may cause the hike in pH.

4.3.2.2. Extent of deprotenisation and demineralization

Deprotenisation from shrimp shell was brought about by the activity of neutral protease of *Bacillus subtilis* (Lauer *et al.*, 2000; Bustos and Healy, 1994). Yang *et al.* (2000) found that optimum activity of protease produced by *Bacillus*

Fig. 4.18. Changes in pH during fermentation of shrimp shell using *Bacillus subtilis*



subtilis takes place at 30^0 C and pH of 6. In the present work also, it showed maximum proteolytic activity of 10.6875 g tyrosine/g protein after 48 hrs of fermentation when the pH of the liquor was 5.9. The proteolytic activity remained fairly constant for 5-6 days during fermentation (Fig. 4.19). These changes were clearly supported by the reports of Allagheny *et al.* (1996) and Sarkar *et al.* (1993).

During fermentation, when the available carbohydrate source was utilized by the microorganisms for their growth, acid was released and this was responsible for calcium solubilisation from the shrimp shell (Rao, 2000). As the calcium ion concentration increased in the fermenting media, proteolytic activity showed a decreasing trend. Tsuru *et al.* (1964) reported that calcium is recognized to prevent protease autolysis.

Table 4.30 shows the protein and ash content of the fermented residue. About 84% of protein and 72% of minerals were removed from the shrimp shell at the end of fermentation.

4.3.2.3. Mild chemical treatment of fermented residue for the production of chitin.

To produce chitin of standard qualities, residual protein and ash content of the fermented residue has to be removed by mild acid and alkali treatments. Different concentrations of hydrochloric acid and sodium hydroxide were tried to remove ash and protein respectively (Figs 4.20 and 4.21)

Considering the statistical significance ($P<0.05$) in the extent of demineralization trials, we have taken 0.8N HCl for the demineralization study. Similarly, among different alkali concentrations tried, 0.6M NaOH was used for the deproteinisation ($P<0.05$).

Many previous works reported the chemical treatment of the fermented residue to remove the residual protein and ash (Cira *et al.*, 2002; Shahidi and Synowiecki, 1991; No *et al.*, 1989). The optimal concentrations of the acid and

Table 4.30. Protein and ash content of the shell residue obtained after *Bacillus subtilis* fermentation

Particulars	% of protein content	% of ash content
Raw material	38.10±2.15	18.53±0.89
Fermented residue	6.09±0.42	5.19±0.36

Fig. 4.19. Changes in proteolytic activity during fermentation with *Bacillus subtilis*.

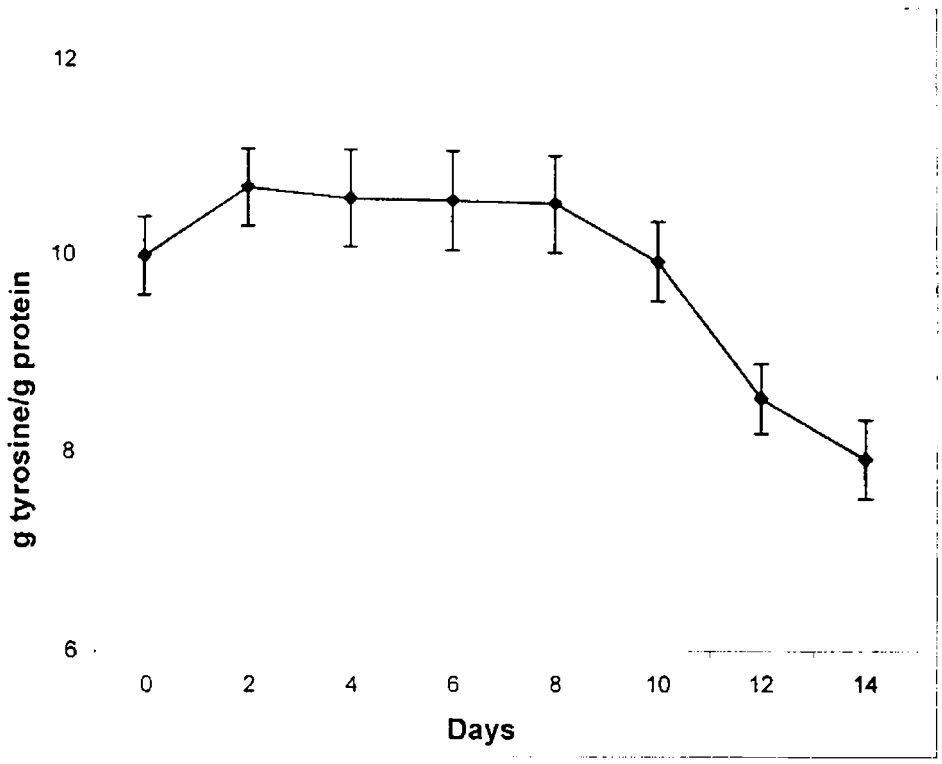


Fig. 4.20. Percentage of ash content in the fermented residue treated with different concentrations of HCl.

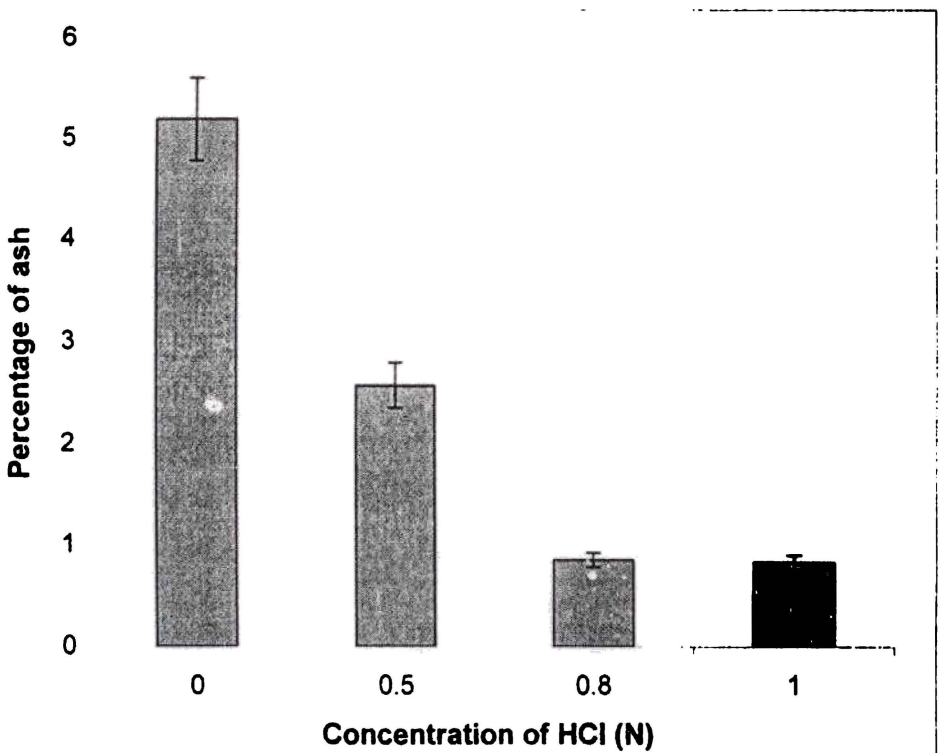
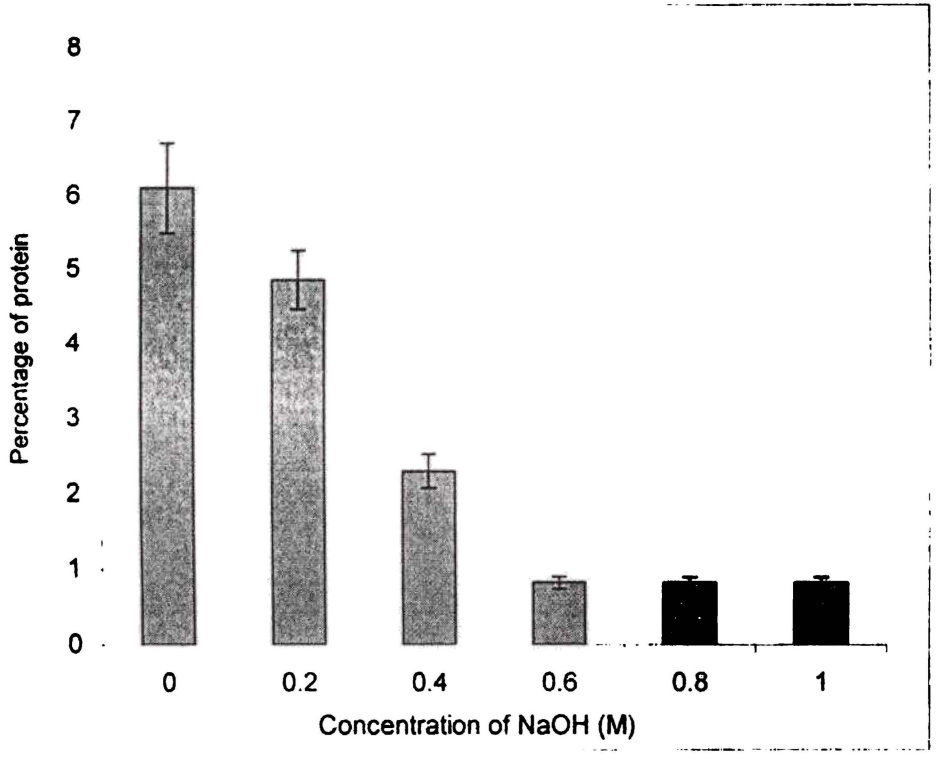


Fig. 4.21. Percentage of protein content in fermented residue treated with different concentrations of alkali



alkali required depends on the residual protein and ash contents. This was dependent on the type of the raw material, type of the starter culture, fermentation time etc.

Chitosan was prepared from the chitin produced.

4.3.2.4. Characteristics of chitin and chitosan prepared from the fermented residue

Table 4.31 presents the characteristics of the chitin prepared. The chitin content of the product was found to be 94.6%. Mineral content (calcium, sodium, potassium and magnesium) in the chitin prepared from the shrimp shell is shown in Fig. 4.22. From the figure, it is clear that calcium content is more than other minerals in chitin. This is due to the fact that the main constituent of minerals in shrimp shell is calcium carbonate (Legarreta *et al.*, 1996). Mild acid treatment after fermentation, reduced the mineral content to permissible limit in chitin prepared. One of the factors determining the good quality of chitin is low mineral content (Tolaimate *et al.*, 2003).

FTIR spectra of chitin prepared is depicted in the Fig. 4.23. The spectrum was found to be similar to the commercial chitin (Fig. 4.8). There are no noises of impurities found. Since, it is having better percentage of individual functional groups, the chitin prepared by the bacillus fermentation shoulders with the chitin availed commercially in purity aspect. The percentage of N-acetylation, calculated from the FTIR spectrum, was also found to be remarkable, i.e; 84.4%.

Characteristics of chitosan prepared were given in Table 4.32. Chitosan prepared was found to have good viscosity and high molecular weight. FTIR spectrum of the prepared chitosan was presented in Fig. 4.24 and it was observed to be similar to that of commercial chitosan (Fig. 4.25).

4.3.2.4. Comparison between different microorganisms on the extent of deprotenisation and demineralization

It was found that there was significant difference ($P<0.05$) in the extent of protein removed in samples fermented with *Lactobacillus plantarum* and *Bacillus subtilis* and between *Lactobacillus brevis* and *Bacillus subtilis* (Table 4.33). About 84% protein were removed in samples fermented with *Bacillus subtilis*. Significant analyses were carried out for the extent of demineralization also (Table 4.33). It was found that there was not much significant difference in the extent of demineralization

Table 4.31. Properties of chitin prepared from the fermented residue obtained by *Bacillus subtilis* fermentation

Moisture	7.12± 0.4%
Ash	0.85± 0.04%
Protein	0.81± 0.05%
Chitin	94.61± 0.60%
Appearance	White flakes
% of N-acetylation	84.42± 5.30
pH	7.82± 0.20

Table 4.32. Characteristics of chitosan prepared.

Moisture (%)	7.62±0.53
Ash (%)	0.83±0.06
Appearance	Off white flakes
Acid Insoluble ash	Nil
Solubility (10%) in 1%acetic acid	95%
Viscosity (m Pa.S)	1685±138
Molecular weight ($X10^5$) (Da)	2.62±0.15
Degree of deacetylaton (%)	83±7

Fig. 4.22. Mineral content of the chitin prepared from the shell residue of *Bacillus subtilis* fermentation

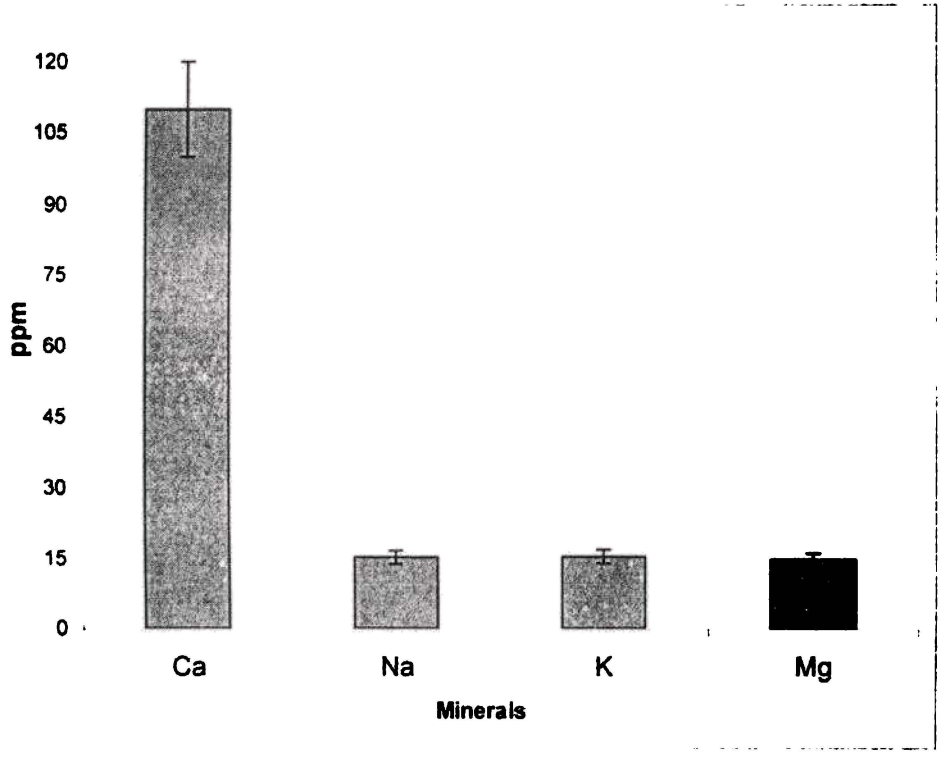


Fig. 4.23. FTIR spectrum of chitin prepared from the shell residue of *Bacillus subtilis* fermentation

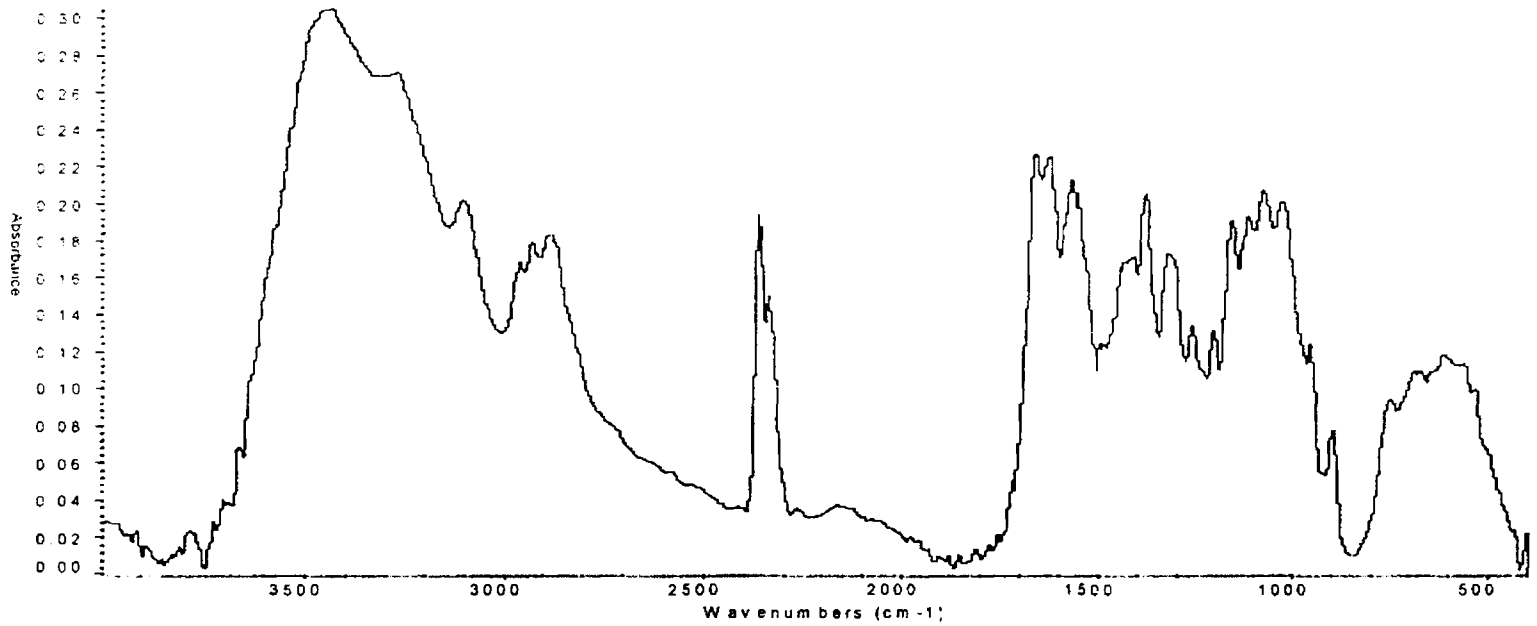


Fig. 4.24. FTIR spectrum of chitosan prepared from *Bacillus subtilis* fermented shrimp shell residue

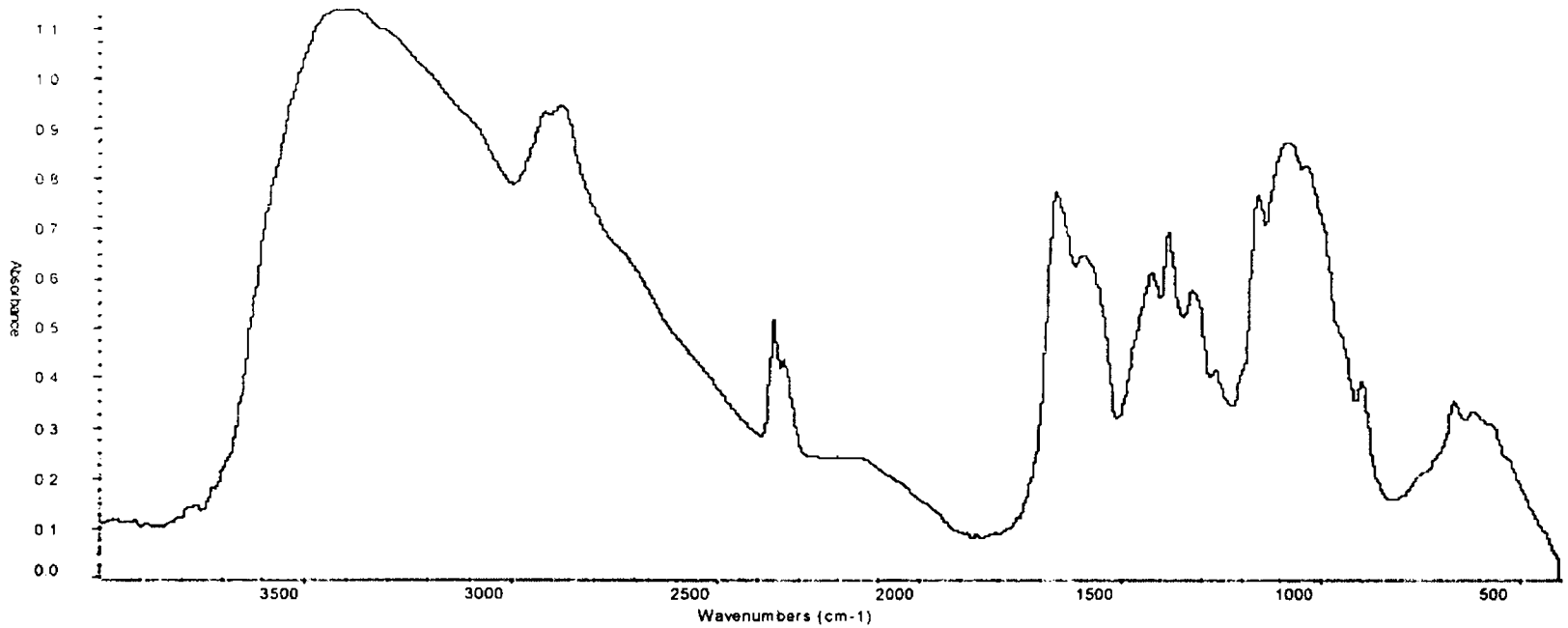


Fig. 4.25. FTIR spectrum of commercial chitosan

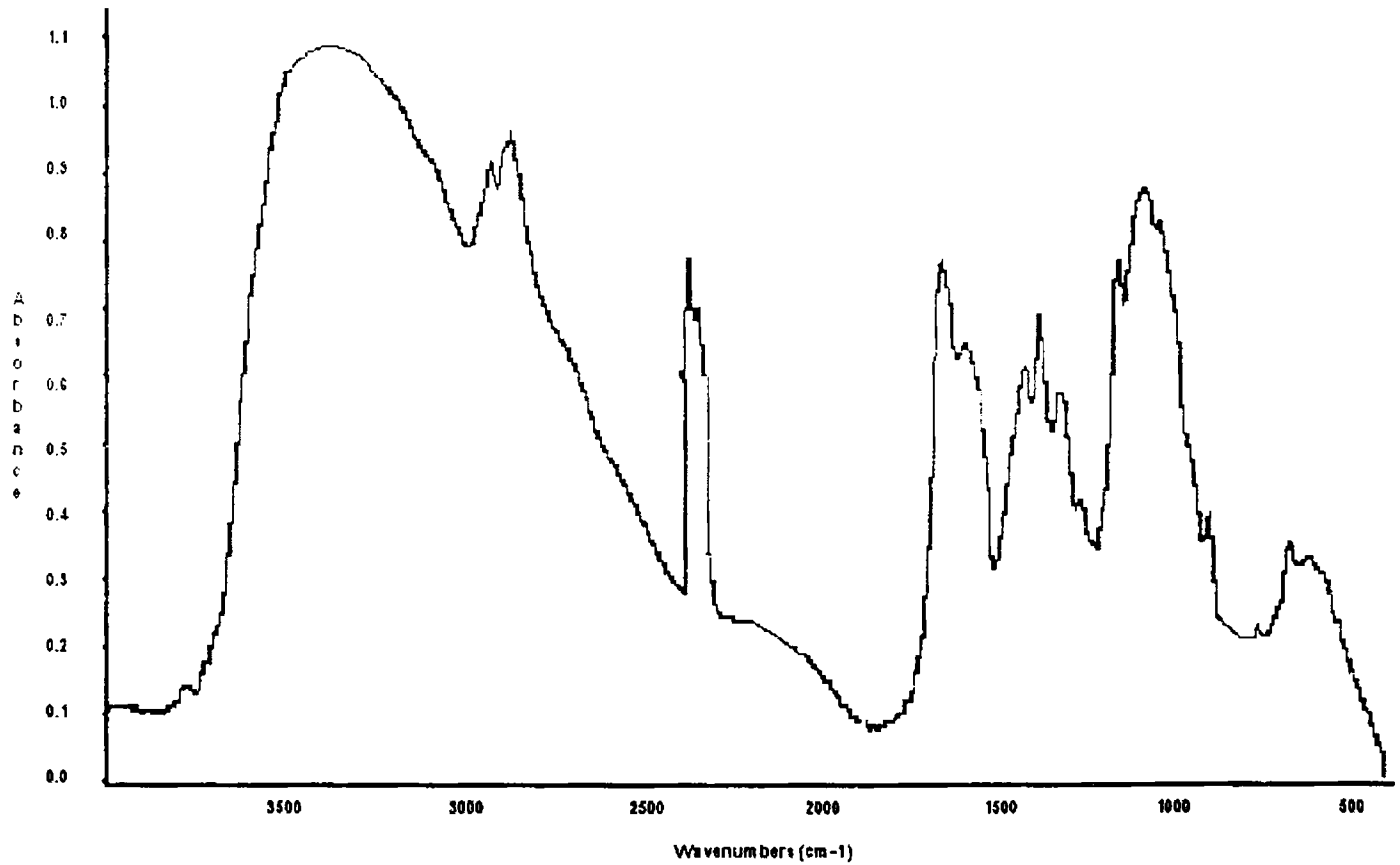


Table 4.33. Comparison of extent of deproteinisation and demineralization of shell fish waste after fermentation with different microorganisms.

Microorganism	Extent of deproteinisation (%)	Extent of demineralisation (%)
Bacillus subtilis	84.02±0.2 [a,b]	71.99±0.63
Lactobacillus plantarum	72.8±5.98 [a]	76.5±6.47
Lactobacillus brevis	68.15±4.83 [b]	67.48±4.12

[a] $P<0.05$ significantly different compared with different microorganisms

[b] $P<0.05$ significantly different compared with different microorganisms

with three different microorganisms. In fermentation trials with *Lactobacillus plantarum* and *Lactobacillus brevis*, initial pH adjustment were done with HCl and lactic acid during the first 5-6 hrs of fermentation to avoid spoilage. So this acid treatment also influenced the demineralization process during fermentation with the two Lactobacillus species. But with Bacillus species, demineralization effected only due to the acid produced by the organism during fermentation.

5. SUMMARY AND CONCLUSION

Since, chemical method, which is used conventionally nowadays, deteriorates the properties of the chitin prepared, biological method of deproteinisation and demineralization for chitin preparation can be adopted as an alternative. This study is an attempt to go deeper to the beneficiaries of fermentation techniques for the preparation of chitin from shrimp shell waste. Efficiency of three different strains of bacteria for the removal of proteins and minerals from the shrimp shell was studied. *Lactobacillus plantarum*, *Lactobacillus brevis* and *Bacillus subtilis* were used for the study.

Fermentation parameters were optimized for the fermentation with all the three strains. Type and quantity of the sugar, quantity of the inoculum, fermentation period etc. were standardized by studying different fermentation parameters like changes in pH, total titrable acidity, changes in proteolytic activity, changes in microbial count, sensory evaluation for spoilage, extent of deproteinisation and extent of demineralization.

In all the studies, it was found that pH first started decreasing with fermentation time accordingly total titrable acidity was increased. This was in line with the increase of proteolytic activity. After reaching the optimum level, proteolytic activity showed a declining trend and parallely pH started increasing as the acid production yielded to low. During fermentation, the bacterial strains utilized the sugar available in the fermenting media and at the end of fermentation; about 87% of the sugar was utilized. From the optimization studies, 20% w/v jaggery broth containing 10^8 CFU/ml *Lactobacillus plantarum* was used for fermentation studies for 15 days. To adjust initial pH adjustment, mild HCl and lactic acid were used for the study. Lactic acid treated samples showed greater extent of demineralization and deprotenisation at the end of fermentation study than hydrochloric acid treated samples. It can be due to the effect of strong hydrochloric acid on the initial microbial count, which directly affects the fermentation process. At the end of fermentation, about 76.5% of ash was removed in lactic acid treated samples and 71.8% in hydrochloric acid treated samples; 72.8% of proteins in lactic acid treated samples and 70.6% in

hydrochloric acid treated samples. Lactic acid treated samples showed greater extent of demineralization and deprotenisation at the end of fermentation study than hydrochloric acid treated samples. The residual protein and ash in the fermented residue were reduced to permissible limit by treatment with 0.8N HCl and 1M NaOH to produce chitin. The properties of chitin and chitosan were studied.

Detailed fermentation study with *Lactobacillus brevis*, was done with 20% w/v jaggery broth for 17 days. All the parameters affecting the fermentation like pH changes, changes in microbial count, changes in proteolytic activity, sensory changes etc were studied. At the end of fermentation, protein remaining in the samples were only 32.48% in HCl treated samples and 31.85% in lactic acid treated samples. About 33.68% of residual ash was present in HCl treated samples and 32.52% in lactic acid treated ones. For conversion to chitin, mild acid and alkali treatments were standardized. The fermented residue was converted to chitin with good characteristics by treatment with 1.2M NaOH and 1N HCl in both HCl treated and lactic acid treated samples. Chitosan prepared from the chitin prepared from the fermented residue was with high molecular weight and high viscosity.

Fermentation with *Bacillus subtilis* was done for 15 days with 20%w/v jaggery broth. From the studies it was found that the proteolytic activity of Bacillus was more compared to Lactobacillus strains. The residual protein content was only 16% and residual ash content was about 28% in the fermented residue. Different trials of mild acid and alkali treatment done to standardize the chemical treatment concentration for the preparation of chitin. 0.8N HCl and 0.6M NaOH was used for the preparation of chitin.

From the studies it was found that *Bacillus subtilis* strain was the most efficient for removing the proteins from the shrimp shell than Lactic acid bacteria. But the extent of demineralization obtained was almost similar in fermentation trials with *Lactobacillus plantarum* and *Bacillus subtilis*. Protein liquor obtained from Lactic acid fermentation was also studied to study the extent of spoilage.

Chitosan with highest viscosity and molecular weight was obtained in *Lactobacillus plantarum* fermentation.

6. REFERENCES

A.O.A.C. *Official methods of analysis*, Association of Official Analytical Chemists. 12th Edition, AOAC International, Washington DC, USA, 1975, pp.75-79.

A.O.A.C. *Official Methods of Analysis*, Association of Official Analytical Chemists, 13th Edition, AOAC International, Washington DC, USA, 1980, pp.41-43.

Abazinge, M.D.A., Fontenot J.P., Allen V.G. Effect of various additives on the preservation and ensiling characteristics of crab waste. *Anim. Sci. Res. Rep. Agr. Exp. Station* 1986; 5: 107-111.

Abazinge, M.D.A., Fontenot, J.P., Allen, V.G. Digestibility, nitrogen utilization, and voluntary intake of ensiled crab waste-wheat straw mixtures fed to sheep. *J. Anim. Sci.* 1994; 72: 565-571.

Adamberg, K., Kask, S., Laht, T.M., Paalme, T. The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. *Int J. Food Microbiol.* 2003; 85: 171– 183.

Adams, M.R., Cooke, R.D., Twiddy, D.R. Fermentation parameters involved in the production of lactic acid preserved fresh glucose substrates. *Int. J. Food Sci. Tech.* 1987; 22: 105-144.

Aguirre, M., Collins, M.D. Lactic acid bacteria and human clinical infection. *J. Appl. Bacterial.* 1993; 75: 95-107.

Albrecht, C., Idler, F., Venus, J., Schade, W. Emsatz von Milchsaurebakterien als Starterkulturen fur die Konsevierung von Grunfutterstoffen. 3. Mitteilung. Testung ausgewahlter. Statue in Modellsilierversuchen. *Wirtscha Oseigene Futter* 1992; 38: 124-130.

Allagheny, N., Obanu, Z.A., Campbell Platt, G., Owens, J.D. Control of ammonia formation during *Bacillus subtilis* fermentation of legumes. *Int. J. Food. Microbiol.* 1996; 29: 321–333.

Alvarez, R.J. A comparative study of the nutritional value of the protein from ensiled fish. *Cuban J. Agric. Sci.* 1972; 6: 219-222.

Ampe, F., Sirvent, A., Zakhia, N. Dynamics of the microbial community responsible for traditional sour cassava starch fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization. *Int. J. Food Microbiol.* 2001; 65: 45– 54.

Andrade, V.S., de Barros, N.B., Fukushima, K., de Campos, G.M.T. Effect of medium components and time of cultivation on chitin production by *Mucor circinelloides (Mucor javanicus* IFO 4570) A factorial study. *Rev. Iberoam Micol.* 2003; 20: 149-153

Anon. Fermented fish products, *Fisheries Report* 100, 1971, FAO, Rome.

Aoyama, Y., Shigeta, AY., Okazaki, T., Hagura, Y., Suzuki, K. Germination and inactivation of bacillus subtilis spores under combined conditions of hydrostatic pressure and medium temperature. *Food Sci. Techol. Res.* 2005; 11: 101-105

Atalo, K., Gashe, B.A. Protease production by a thermophilic *Bacillus*species (P-001A) which degrades various kinds of fibrous proteins. *Biotechnol. Lett.* 1993; 15: 1151–1156.

Averbach, B.L. The structure of chitin and chitosan. Report No. MITSG 75-17., *MIT Sea Grant Report* 1975; 12-23

Bacus, J., Brown, W. The lactobacilli: Meat products. In: Gilliland S.E. (Ed.), *Bacterial starter cultures for foods.* CRC Press Inc., Boca Raton, 1985, pp 47-72.

Banos, S. B., Lauzardo, A. N. H., Velazquez-del Valle, M. G., Hernandez-Lopez, M., Barka, E. A., Bosquez-Molina, E., Wilson, C. L. Fishery wastes. *Crop Prod.* 2006; 25: 108-112.

Bautista , J., Jover, M., Gutierrez, J.F., Corpas, R., Cremades, O., Fontiveros, E., Iglesias, F., Vega, J. Preparation of crayfish chitin by in situ lactic acid production. *Process Biochem.* 2001; 37: 229–234.

Beddows, C. Fermented fish and fish products. In: B. Wood (Ed.) *Microbiology of fermented food*, Elsevier Applied Science Publishers, New York, 1985, 2, pp. 55-59.

ben Omar, N., Ampe, F., Raimbault, M., Guyot, J.P., Tailliez, P. Molecular diversity of lactic acid bacteria from cassava sour starch (Colombia). *Syst. Appl. Microbiol.* 2000; 23: 285– 291.

Benjakul, S. Visessanguan, W., Tanaka, M., Ishizaki, S., Suthidhan, R., Sungpech, O. Effect of chitin and chitosan on gelling properties of surimi from barred garfish (*Hemiramphus oar*). *J. Sci. Food. Agric.* 2001; 81: 102-108.

Benkerroum, N., Ghouati, Y., Ghalfi, H., Elmejdoub, T., Roblain, D. Jacques, P., Thonart, P. Biocontrol of Listeria monocytogenes in a model cultured milk (Iben) by in situ bacteriocin production from Lactococcus lactis ssp. Lactis. *Int. J. Dairy Technol.* 2002; 55: 145–151.

Beresford, T. P., Fitzsimons, N. A., Brennan, N. L., Cogan, T. M. Recent advances in cheese microbiology. *Int. Dairy J.* 2001; 11: 259–274.

Bie, R., Sjostrom, G. Autolytic properties of some lactic acid bacteria used in cheese production. Part II. Material and methods. *Milchwissenschaft* 1975; 30: 653-657.

Blanco, J. Produccio´n de a´cido la´ctico a partir de almidon de patata mediante un proceso de fermentacio´n feed-batch. Ph.D. Thesis. Universidad de Madrid, 1986; pp. 36-42.

Bocker, G., Stolz, P., Hammes, W. P. Neue Erkenntnissezum Okosystem Sauerteig und zur Physiologie der sauerteigtypischen Stamme

Lactobacillus sanfrancisco und Lactobacillus pontis. *Getreide Mehl und Brot* 1995; 49: 370–374.

Brine, C.J., Austin,P.R. Chitin variability with species and method of preparation. *Comp. Biochem. Physiol.* 1981; 69B: 283– 286.

Brookes, R.M., Buckle, A.E. Lactic acid bacteria in plant silage. In: B.J.B. Wood (Ed.) *The Lactic Acid Bacteria*, Elsevier Applied Science, London, 1992; 1: pp. 363-386.

Broussignac, P. Chitosan, a natural polymer not well known by the industry. *Chim. Ind. Genie Chim.* 1968; 99: 1241–1247.

Brown, M.H., Booth, J.R. Acidulants and low pH. In: N.J. Russell, G.W. Gould (Eds) *Food Preservatives*. Blackie, Glasgow, 1991; pp. 22-43.

Bucharles, C., Girard, J. P., Sirami, J., Pascal, G. Prawn shell waste utilization. *Sci. Aliment.* 1984; 4: 137-142.

Bustos, R.O., Healy, M.G. Microbial extraction of chitin fromprawn shell waste. In: *Second InternationalSymposium on En_ironmental Biotechnology.* Biotechnology.1994; pp. 15–25.

Bustos, R.O., Michael, H. Microbial deproteinisation of waste prawn shell. Institution of Chemical Engineers Symposium Series, Institution of Chemical Engineers, Rugby, England 1994; pp. 13–15.

Buyong, N., Kok, J., Luchansky, J. B. Use of a genetically enhanced, pediocin-producing starter culture, Lactococcus lactis subsp. lactis MM2177, to control Listeria monocytogenes in Cheddar cheese. *Appl. Environ. Microb.* 1998; 64: 4842–4845.

Callewaert, R., Hugas, M., De Vuyst, L. Competitiveness and bacteriocin production of Enterococci in the production of Spanish-style dry fermented sausages. *Int. J. Food Microbiol.* 2000; 57: 33–42.

Campbell-Platt, G. African locust bean *(Parkia* species) and its West African fermented food product,dawadawa. *Ecol. Fd. Nutrit.,* 1980; 9: 123-132.

Caplice, E., Fitzgerald, G.F. Food fermentations: role of microorganisms in food production and preservation. *Int. J. Food Microbiol.* 1999; 50: 131–149.

Castro, S., Peyronel, D.V., Cantera, A.M.B. Proteolysis of Whey Proteins by a Bacillus subtilis Enzyme Preparation. *Int. Dairy J.* 1996; 6: 285-294.

Cervantes, A. Ensilaje y harina de pescado en la ceba de cerdos. *Informe de Tema MINAG*, La Habana, 1979; pp. 75-82.

Champomier-Verges, M.C., Maguin, E., Mistou ,M.Y., Angladec, P., ois Chich, J.F. Lactic acid bacteria and proteomics: current knowledge and perspectives. *J. Chromatogr B* 2002; 771: 329–342.

Chen, G., Russell, J.B Transport of glutamine by *Streptococcus bovis* and conversion of glutamine to pyroglutamic acid and ammonia. *J. Bacterial.* 1989; 171: 2981-2985.

Chen, L., Coolbear, T., Daniel, R.M. Characteristics of proteinases and lipases produced by seven Bacillus sp. isolated from milk powder production lines. *Int. Dairy J.* 2004; 14: 495–504.

Chopra, A.K., Mathur, D.K. Purification and characterization of heat-stable proteases from Bacillus stearothermophilus RM-67. *J. Dairy Sci.* 1985; 68: 3202–3211.

Cira, L.A., Huerta, S., Hall, G.M., Shirai, K. Pilot scale lactic acid fermentation of shrimp wastes for chitin recovery. *Process Biochem.* 2002; 37: 1359-1366

Collins, M. D., Rodrigues, U. M., Ash, C., Aguirre , M., Farrow, J. A. E., Martinez-Murcia, A., Phillips, B. A., Williams, A. M., Wallbanks, S. Phylogenetic analysis of the genus Lactobacillus and related lactic acid bacteria as

determined by reverse transcriptase sequencing of 16s rRNA. *FEMS Microbiol. Lett.* 1991; 77: 5-12.

Connell, J.J. Methods of dressing and selecting for quality. In : *Control of fish quality*. Fishing News (Books) Ltd., England, 1980; pp. 107-135.

Conway, E.J. In: *Microdiffusion Analysis and Volumetric Error*, 5th Edition, Parch, Crosbey and Lockwod and Sons Ltd., London, 1950; pp. 467-472.

Coughlin, R.W., Deshaies, M.R., Davis, E.M. Chitosan in crab shell wastes purifies electroplating wastewater. *Environ. Prog.* 1990; 9: 35-39

Cox, R.P., Thomsen, J.K. Computer-aided identification of lactic acid bacteria using the API 50 CHL system. *Lett. Appl.Microbiol.* 1990; 10: 257–259.

Crini, G. Fermentation of shell waste. *Bioresource Technol.* 2006; 97: 1061-1068

Daeschel, M.A. Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technol.* 1989; 1: 164-167.

Dako, E., El Soda, M., Vuillemard, J.C., Simard, R.E. Autolytic properties and aminopeptidase activities of lactic acid bacteria. *Food Res. Int.* 1995; 28: 503-509.

Dapkevicus, M.D.L.E., Batista, I., Nout, M.J.R., Rombouts, F.M., Houben, J.H. Lipid and protein changes during the ensilage of blue whiting by acid and biological methods. *Food Chem.* 1998; 63: 97-102.

de Man, J.C., Rogosa, M., Sharpe, M.E. A medium for the cultivation of lactobacilli. *J. Appl. Microbiol.* 1960; 23: 130-135.

de Vuyst, L., Vandamme, E.J. Antimicrobial potential of lactic acid bacteria. In: *Bactetiocins of Lactic Acid Bacteria*, Blackie Academic and Professional, London, 1994; pp. 91-143

den Brinker, C., Rayner, C., Kerr, M. In: Investigation of biogenic amines in fermented fish and fish products. Public Health Division, Victorian Government Department of Human Service, Australia. 1996; pp. 1-5

Dodane, V., Vilivalam, V.D. Pharmaceutical applications of chitosan. In: *Research Focus*, Elsevier Science, London, 1998; 1: 246-253.

Dominguez, P.L. Utilización de desperdicios alimentarios y de subproductos industriales agropecuarios y de la pesca en la alimentación del cerdo. In: *Alimentación Porcina No-Convencional CIDA*, La Habana, 1988; pp. 67-72.

Dong, F.M., Fairgrieve, W.T., Skonberg, D.I., Rasco, B.A. Preparation and nutrient analyses of lactic acid bacterial ensiled salmon viscera. *Aquaculture* 1993; 109: 351-366.

Dunne, C., Murphy, L., Flyin, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrisey, D., Thorton, G., Fitzgerald, G., Daly, C.,Kiely, B., Quigley, E. M. M., O'Sullivan, G. C., Shanahan, F., & Collins, K. Probiotics: From myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antoine van Leeuwenhoek* 1999; 76: 279–292.

Edwards, R. A., Dainty, R. H., Hibbard, C. M., Ramantanis, S. V. Amines in fresh beef of normal pH and the role of bacteria in changes in concentration observed during storage in vacuum packs at chill temperatures. *J. Appl. Microbiol.* 1987; 63: 427-434.

Egorov, N.S., Vybornykh, S.N., Kreier, V.G., Loriya, Z.K. Spore-for ming exoprotease from Bacillus licheniformis strain 28KA.Translated by Plenum Publishing Corporation from. *Priladnaya Biokhimiya i Mikrobiologiya* 1982; 17: 882–889.

Eitenmiller, R. Orr, J., Wallis, W. Histamine formation in fish: Microbiological and biochemical conditions. In: R. Martin (Ed.) Chemistry and Biochemistry of Marine food products. AVI, Connecticut, Japan, 1980; pp. 39-50.

den Brinker, C., Rayner, C., Kerr, M. In: Investigation of biogenic amines in fermented fish and fish products. Public Health Division, Victorian Government Department of Human Service, Australia. 1996; pp. 1-5

Dodane, V., Vilivalam, V.D. Pharmaceutical applications of chitosan. In: *Research Focus*, Elsevier Science, London, 1998; 1: 246-253.

Dominguez, P.L. Utilización de desperdicios alimentarios y de subproductos industriales agropecuarios y de la pesca en la alimentación del cerdo. In: *Alimentación Porcina No-Convencional CIDA*, La Habana, 1988; pp. 67-72.

Dong, F.M., Fairgrieve, W.T., Skonberg, D.I., Rasco, B.A. Preparation and nutrient analyses of lactic acid bacterial ensiled salmon viscera. *Aquaculture* 1993; 109: 351-366.

Dunne, C., Murphy, L., Flyin, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrisey, D., Thorton, G., Fitzgerald, G., Daly, C.,Kiely, B., Quigley, E. M. M., O'Sullivan, G. C., Shanahan, F., & Collins, K. Probiotics: From myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antoine van Leeuwenhoek* 1999; 76: 279–292.

Edwards, R. A., Dainty, R. H., Hibbard, C. M., Ramantanis, S. V. Amines in fresh beef of normal pH and the role of bacteria in changes in concentration observed during storage in vacuum packs at chill temperatures. *J. Appl. Microbiol.* 1987; 63: 427-434.

Egorov, N.S., Vybornykh, S.N., Kreier, V.G., Loriya, Z.K. Spore-for ming exoprotease from Bacillus licheniformis strain 28KA.Translated by Plenum Publishing Corporation from. *Priladnaya Biokhimiya i Mikrobiologiya* 1982; 17: 882–889.

Eitenmiller, R. Orr, J., Wallis, W. Histamine formation in fish: Microbiological and biochemical conditions. In: R. Martin (Ed.) Chemistry and Biochemistry of Marine food products. AVI, Connecticut, Japan, 1980; pp. 39-50.

EnarPb, G., El-Essawyb, A.A., Uyttendaele, M., Debeverea, J. Antibacterial activity of *Lactobacillus plantarum* UGI isolated from dry sausage: characterization,production and bactericidal action of plantaricin UG 1. *Int. J. Food Microbiol.* 1996; 30: 189-215

Evers, D.J., Carroll, D.J. Preservation of crab or shrimp waste as silage for cattle. Anim. Feed Sci. Tech. 1996; 59: 233-244.

Fayolle, P, Picque, D., Corrieu, G. Monitoring of fermentation processes producing lactic acid bacteria by mid-infrared spectroscopy . *Vib. Spectrosc.* 1997; 14: 247-252.

Fenlon, D.R., Wilson, J., Donald, S. The use of bacteriocin producing *Pediococcus acidilactici* as a silage inoculant to control contamination by listeria. In: O' Kiely, P., O' Connell, M., Murphy, J. (Eds) *Silage Research*, Proceedings of the Tenth International Conference on Silage Research, Dublin City University, Ireland, 1993; pp. 80-81.

Figueroa, C., Davila, A.M., Pourquie, J. Lactic acid bacteria of the sour cassava starch fermentation. *Lett. Appl. Microbiol.* 1995; 21: 126–130.

Figueroa, C., Davila, A.M., Pourquie, J. Original properties of ropy strains of Lactobacillus plantarum isolated from the sour cassava starch fermentation. *J. Appl. Microbiol.* 1997; 82: 68–72.

Fisher, E.H., Stein, E.A. In: P.D. Boyer, H. Lardy, M. Myrback (Eds.) *The Enzymes*, 2nd Edition, Academic Press, New York, 1960; pp. 313-318.

Fisher, E.H., Sumerwell, W.N., Junge, J.M., Stein, E.A. In: Proceedings of the VIII Symposium of the IV International Congress of Biochemistry, Vienna, Pergamon Press, London, 1958; pp. 23-28.

Fitzsimons, A., Duffner, F., Curtin. D.. Brophy. G., O' Kiely, P., O' Connell. M. Assessment of *Pediococcus acidilactici* as a potential silage inoculant. *Appl. Environ. Microb.* 1992; 58: 3047-3052.

Fleming, H.P., Mc Feeters, R.F., Daeschel, M.A. The lactobacilli, pediococci and leuconostots:vegetable products. In: S.E. Gilliland (Ed.) *Bacterial starter cultures for foods*, CRC Press Inc., Boca Raton, 1985; pp. 97- 118.

Foegeding, P.M., Thomas, A.B., Pilkington, D.H., Klaenhammer, T.R. Enhanced control of *Listeria monocytogenes* by in situ-produced pediocin during dry fermented sausage production. *Appl. Environ. Microb.* 1992; 58: 884–890.

Gagne, N, Simpson, B.K. Use of proteolytic enzymes to facilitate recovery of chitin from shrimp wastes. *Food Biotechnol.* 1993; 7: 253–263.

Giraffa, G. Enterococcal bacteriocins: their potential as anti- Listeria factors in dairy technology. *Food Microbiol.* 1995; 12: 291–299.

Grant. M.A., Harrison, J.A., Rink, S., Loney. K.A. Novel use of bacteria from pickle fermentation as silage additive. *J. Dairy Sci.* 1994; 77: 3388-3400.

Green, S., Wiseman, J., Cole, D.J.A. Fish silage in pig diets. *Pig News and Information* 1983; 4: 269-273.

Guillou, A., Khalil, M., Adambounou, L. Effects of silage preservation on astaxanthin forms and fatty acid profiles of processed shrimp. *Aquaculture* 1995; 130: 351-360.

Haard, N.F., Kariel, N., Herzberg, G., Feltham, L.A.W., Winter, K. Stabilization of protein and oil in fish silage for use as a ruminant feed supplement. *J. Sci. Food Agri.* 1985; 36: 229-241.

Halasz, A., Barath, A., Simon-Sarkadi, L., Holzapel, W. Biogenic amines and their production by micro-organisms in food - Review. *Trends Food Sci. Tech.* 1994, 42-49.

Hall, G.M., de Silva, S. Lactic acid fermentation of shellfish *(Penaeus monodon)* waste for chitin recovery. In: C.J. Brine, P.A. Sandford, J.P. Zikakis

(Eds) *Advances in Chitin and Chitosan*. Elsevier Applied Science, London, 1992; pp. 633-638.

Hall, G.M., de Silva, S. Recovery of chitin. *Biotechnology* 1994, 633–638.

Hall, G.M., Reid, C.L. Scale up of lactic acid fermentation process for the recovery of chitin from prawn waste. In: M.B. Zakaria, W.M. Wan Muda, M.P. Abdullah (Eds) *Chitin and Chitosan - TheVersatile Environmentally Friendly Modern Materials*. National University of Malaysia, Kuala Lumpur, 1994; pp. 48-51.

Hammes, W. P., Bantleon, A., Min, S. Lactic acid bacteria in meat fermentation. *FEMS Microbiol. Rev.* 1990; 87: 165-174.

Hammes, W.P., Weiss, N., Holzapfel, W.P. (1991). The genera Lactobacillus and Carnobacterium. In: A. Balows, H.G. Trtiper, M. Dworkin, W. Harder, K.H. Schleifer (Eds) *The Prokaryotes. A Handbook on the Biology of Bacteria*. Springer, New York, 1991; pp. 1535-1594.

Harris, L.J. The microbiology of vegetable fermentations. In: B.J.B. Wood (Ed.) *Microbiology of fermented foods*. Blackie Academic & Professional, London, 1998; 1: pp. 45–72.

Harris, L.J., Fleming, H. P.; Klaenhammer, T. R. Novel paired starter culture system for sauerkraut, consisting of a nisin-resistant Leuconostoc mesenteroides strain and a nisin producing *Lactococcus lactis* strain. *Appl. Environ. Microb.* 1992; 58: 1484–1489.

Hassan, T.E, Heath, J.L. Biological fermentation of fish waste for potential use in animal and poultry feeds. *Agricult. Wastes* 1986; 15:1–15.

Healy, M.G, Romo, C.R, Bustos, R. Bioconversion of marine crustacean shell waste. *Resour. Conserv. Recycling* 1994; 11: 139-147.

Hirano, S. Chitin biotechnology applications. *Biotechnol. Ann. Rev.* 1996; 2: 237-258.

Hodge, J.E., Hofreiter, B.T. Determination of reducing sugars and carbohydrates. In: R.L. Whistler, M.L. Wolfram (Eds). *Methods in Carbohydrate Chemistry.* Academic Press, New York, 1962; 1: pp. 380-394

Holzapfel, W.H., Geisen, R., Schillinger, U. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Int J. Food Microbiol.* 1995; 24: 343-362.

Horner, W.F.A. Preservation of fish by curing (drying, salting and smoking). In: G.M. Hall (Ed.) *Fish Processing Technology.* Blackie Academic and Professional, United Kingdom, 1997; 2: pp. 32-38

Hossain, M.I. Study on conservation of shrimp waste to environmental friendly products. B.Sc Thesis, BGE Discipline, Khulna university, Khulna, Bangladesh, 2003; pp. 23-29

Hugas, M., Garriga, M., Aymerich, M. T., Monfort, J. M. Inhibition of Listeria in dry fermented sausages by the bacteriocinogenic Lactobacillus sake CTC494. *J. Appl. Bacteriol.*1995; 79: 322–330.

Hugas, M., Garriga, M., Aymerick, T., Monfort, J.M. Biochemical characterization of lactobacilli from dry fermented sausages. *Int. J. Food Microbiol.* 1993; 18: 107-113

Hugas, M., Monfort, J.M. Bacterial starter cultures for meat fermentation. *Food Chem.* 1997; 59: 547-554.

Huttunen, E., Noro, K., Yang, Z. Purification and Identification of Antimicrobial Substances Produced by Two *Lactobacihs casei* Strains. *Int. Diary J.* 1995; 5: 503-513.

Idler, C., Idler, F., Venus, J., Schade, W. Emsatz von Milchsauerebakterien als Starterkulturen fur die Konscrvierung yon Grunfutterstoffen. 5. Ergehnisse znr Verdaulichkeit mikrobiell konservierten I:utters, *Wittschaflseigene Futter* 1994; 40: 40-47.

Illum, L. Chitosan and its use as a pharmaceutical excipient. *Pharm. Res.* 1998; 15: 1326-1331.

Inayara, C.A.L., Rose, L.M., Beatriz, M.B., Alvaro, C.N., Regina, M.D.N., Marc-Andre, L., Carlos A.R. Lactic acid bacteria and yeasts associated with spontaneous fermentations during the production of sour cassava starch in Brazil. *Int. J. Food Microbiol.* 2005; 105: 213– 219.

Jayakumar, R., Reis, R.L., Mano, J.F. Chemistry and Applications of Phosphorylated Chitin and Chitosan. *e-Polymers* 2006; 35: 1-16.

Kamaly, K., Marth, E. Enzyme activities of lactic streptococci and their role in maturation of cheese: A review. *J. Dairy Sci.* 1989; 72: 1945-1966.

Kandler, O. Carbohydrate metabolism in lactic acid bacteria. *Ant. V. Leeuwenhoek* 1983; 49: pp. 209–224.

Kandler, O., Weiss, N. Regular, non-sporing Gram-positive rods. In: P. H. A. Sneath, N. Mair, M. E. Sharpe, J. G. Holt (Eds), *Bergey's Manual of Systematic Bacteriology*. William and Wilkins, Baltimore, 1986; 2: pp. 1208–1234.

Khalid, N., Marth, E. Purification and partial characterization of a propyl-dipeptidyl aminopeptidase from *Lactobacillus helveticus* CNRZ 32. *Appl. Environ. Microb.* 1991; 56: 381-388.

Kiers, J.L., Van laeken, A.E.A., Rombouts, F.M., Nout, M.J.R. In vitro digestibility of *Bacillus* fermented soya bean. *Int. J. Food Microbiol.* 2000; 60: 163–169.

Kim, Y.K., Bae, J.H., Oh, B.K., Lee, W.H., Chio, J.W. Enhancement of proteolytic activity excreted from Bacillus stearothermophilus for a thermophilic aerobic digestion process. *Bioresource Technol.* 2002; 82: 157-164.

Klaenhammer, T.R. Bacteriocins of lactic acid bacteria. *Biochemie* 1988; 70: 337-349.

Klijn, N., Weerkamp, A.H., de Vos, W.M. Detection and characterization of lactose-utilizing Lactococcus spp. in natural ecosystems. *Appl. Environ. Microb.* 1995, 61, 788–792.

Knachel, S. Mikrobiell fermentering af fisk ved hjelp av naturligt forekommende laktobaciller.Hovedoppgave, Fiskeriministeriets Forsogslaboratorium, Kovenhavn Univ. Quoted in Raa, J., A.Gildberg and T. Strom. Silage production: Theory and practice. In: D.A. Ledward, A.J. Taylor, R.A. Lawrie (Eds) *Upgrading Waste for Feed and Food*, Butterworths, London, 1981; pp. 117-132.

Kohlmann, K. L., Nielsen, S. S., Steenson, L. R., Ladisch, M. R. Production of proteases by psychrotrophic microorganisms. *J. Dairy Sci.* 1991; 74: 3275–3283.

Kweon, D.K., Song, S. B., Park, Y.Y. Shell waste utilization. *Biomaterials* 2003; 24: 1595-1599.

Lauer, I., Bonnewitz, B., Meunier, A., Beverini, M. New approach for separating *Bacillus subtilis* metalloprotease and a-amylase by affinity chromatography and for purifying neutral protease by hydrophobic chromatography. *J. Chromatogr. B* 2000; 737: 277–284.

Law, B.A., Kolstad, J. Proteolytic systems in lactic acid bacteria. *Antonie Van Leeuwenhoek* 1983; 49, 225-245.

Law, J., Haandrikman, A. Proteolytic Enzymes of Lactic Acid Bacteria Review Article *Int. Dairy J.* 1997; 7:1-11

Legarreta, G.I., Zakaria, Z., Hall, G.M. Lactic acid fermentation of prawn waste: comparison of commercial and isolated starter culture. In *Advances in Chitin Science*. Jacques Andre, Lyon, France, 1996; 1: pp. 399-402.

Leroy, F., de Vuyst, L. Lactic acid bacteria as functional starter cultures for the food fermentation industry .Article in press. *Trends Food Sci. Technol.* 2003; 15: 67-78.

Lindgren, S. Biosafety aspects of genetically modified lactic acid bacteria in EU legislation. *Int. Dairy J.* 1999; 9: 37-41.

Loner, C., Preve-Akesson, K. Acidification properties of lactic acid bacteria in rye sour doughs. *Food Microbiol.* 1988; 5: 43-58.

Lowry, O.H., Rosenberg, N.J., Far, A.L., Randall, J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 1951; 193: 265-275

Mac kay, L.L., Baldwin, K.A. Applications for biotechnology: present and future improvements in lactic acid bacteria- A review. *FEMS Microbiol. Rev.* 1990; 87: 3-14.

Macleod, G.S., Collett, J.H., Fell, J.T. The potential use of mixed films of pectin, chitosan and HPMC for bimodal drug release. *J. Control. Release* 1999, 58, 303-310.

Madhavan, P. Chitin and chitosan and their novel applications. In: *Popular Science Lecture Series*. Central Institute of Fisheries Technology, Cochin, India, 1992; pp. 6-7.

Madigan, M., Martinko, J. In: *Brock Biology of Microorganisms.* Prentice Hall, 2005; 11: pp. 258-262.

Marine-Font, A., Vidal-Carou, M.C., Izquierdo-Pulido, M. , Venciana-Nogues, M., Hernández-Jover. T.T. Les amines biogénes dans les aliments: leur signification, leur analyse *Ann. Fals. Exp. Chim.* 1995; 88: pp. 11-140.

Martin, A. M. Lactic acid fermentation-aided biomass conversion. *WREC* 1996,c; 10: 942-945

Martin, A.M. Fermentation processes for the production of lactic acid. In: F. Bozoglu (Ed.) *Proceedings of the NATO Advanced Study Institute on Current Advances in Genetics, Metabolism and Application of Lactic Acid Bacteria*. Springer-Verlag, Berlin, 1996 a; pp.123-131.

Martin, A.M. Role of lactic acid fermentation in bioconversion of wastes. In: F. Bozoglu (Ed.) *Proceedings of the NATO Advanced Study Institute on Current Advances in Genetics, Metabolism and Application of Lactic Acid Bacteria*. Springer-Verlag, Berlin, 1996 b; pp. 510-514.

Martin, A.M., Bemister, P.L. Use of peat extract in the ensiling of fisheries wastes. *Waste Manage. Res.* 1994; 12: 467-479.

Martino, A.D., Sittinger, M., Risbud, M.V. Chitosan: A versatile biopolymer for orthopaedic tissue-engineering. *Biomaterials* 2005; 26: 5983-5990.

Masson, F., Eclache, L., Compte, T., Talon, R., Montel, M.C. In: *Proc. 42nd International Congress of Meat Science and Technology.* Matforsk, Lillehammer, Norway, 1996; N16: pp. 546-549.

Meyers, S.P., Benjamin, G. Feeding value of crustacean wastes can be improved through proper silage treatment. *Feedstuffs* 1987; 6: pp. 12-16.

Mi, F.L., Shyu, S.S., Lin, Y.M., Wu, Y.B., Peng, C.K., Tsai, Y.H. Chitin/PLGA blend microspheres as a biodegradable drug delivery system: a new delivery system for protein. *Biomaterials* 2003, 24, 5023-5036.

Mills, O.E., Thomas, T.D. Release of cell wall associated proteinase (s) from lactic streptococci. *N.Z. J. Dairy Sci. Technol* 1978; 13: 209-215.

Montel, M.C., Masson, F., Talon, R. Bacterial Role in Flavour Development. *Meat Sci.* 1998; 49, S111-S 123.

Moreno-Arribas, M.V., Carmen Polo, M., Jorganes, F., Munoz, R. Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *Int. J. Food Microbiol.* 2003; 84: 117– 123.

Morris, D.R., Jorstad, C.M. Isolation of conditionally putrescine-deficient mutants of Escherichia coli. *J. Bacteriol.* 1970; 101: 731– 737.

Mcueddeb, H., Sanchez, J., Bardot, C., Fick, M. Membrane bioreactor for lactic acid production. *J. Membrane Sci.* 1996; 114: 59-71.

Muzzarelli, R. A. A. Chitosan-based dietary foods. *Carbohydr. Polym.* 1996; 29: 309-316.

Muzzarelli, R.A.A. and Rocchetty, R. The determi nation n of molybdenum in sea water by hot graphite atomic absorption spectrometry after concentration on p-aminobenzylcellulose or chitosan. *Anal. Chim. Acta.* 1973; 64: 371–379.

Naidu, A.S., Bidlack, W.R., Clemens, R.A. Probiotic spectra of lactic acid bacteria (LB). *Crit. Rev. Food Sci. Nutr.* 1999; 38: 13-126.

Nakano, M.M., Zuber, P. Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*)". *Annu. Rev. Microbiol.* 1998; 52: 165-190.

Nettles, C.G., Barefoot, S.F. Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria. *J. Food Protect.* 1993; 56: 338-358.

No, H.K., Meyers, S.P., Lee, K.S. Isolation and characterisation of chitin from crawfish shell waste. *J. Agr. Food Chem.* 1989; 37: 575-579.

Noirot, P. Replication of the Bacillus subtilis chromosome. In: P. Graumann (Ed.) *Bacillus: Cellular and Molecular Biology.* Caister Academic Press, 2007; pp. 123-128.

Oberman, H., Libudzisz, Z. Fermented milks. In: B.J.B. Wood (Ed.) *Microbiology of fermented foods*. Blackie Academic & Professional, London, 1998; 1: pp. 308– 350.

Odunfa, S.A., Dawadawa, P. In: N.R. Reddy, M.D. Pierson, D.K. Salunkhe (Eds) *Legume-based Fermented Foods*. CRC Press, Boca Raton, 1986; pp. 173- 189.

Ohta, T., Natto, K. In: N.R. Reddy, M.D. Pierson, D.K. Salunkhe (Eds) *Legume-based Fermented Foods*. CRC Press, Boca Raton, 1986; pp. 85-93.

Ornum, J.V., Shrimp waste - must it be wasted? *Infofish International* 1992, 6, 48-52.

Fagbenro, O.A & Bello-Olusoji, O.A (1997) Preparation, nutrient composition and digestibility of fermented shrimp head silage Food Chemistry, Vol. 60, No. 4, pp. 489-493

Pahlow, G. Role of microflora in forage conservation. In: G. Pa Mow, H. Honig (Eds) *Proceedings of a Conference on Forage Conservation Towards 2000*. Braunschweig, Germany, 1991; pp. 26-36.

Paterson, G.V., Madie, P., Blackmore, D.K. Veterinary aspects of meat quality. Publication No. 135.Veterinary continuing education, Massey University, Palmerston North, New Zealand, 1990; pp 219-232.

Payot, T., Chemaly, Z., Fick, M. Lactic acid production by *Bacillus coagulans*—Kinetic studies and optimization of culture medium for batch and continuous fermentations. *Enzyme Microb. Tech.* 1999; 24: 191–199.

Philip, M., Nair, K.G.R. Ensilation of shrimp waste by *Lactobacillus fermentum*, *Fish. Technol.* 2006; 43: 59-64.

Pintado, J., Guyot, J. P., Raimbault, M. Lactic acid production from mussel processing wastes with an amylolytic bacterial strain. *Enzyme Microb. Tech.* 1999; 24: 590-598.

Poffe^{I}, R., Mertens, W. Heat stable proteases of psychrotrophic bacteria isolated from cooled raw milk. *Appl. Microbiol. Biot.* 1988; 27: 437–442.

Pope, C.G., Stevens, M.F. The determination of amino nitrogen using a copper method. *Biochemic. J.* 1939; 33 : 1070-1075.

Prabaharan, M., Mano, J. F. Chitosan derivatives bearing cyclodextrin cavitiesas novel adsorbent matrices. *Carbohyd. Polym.* 2006, 63, 153.

Prasad, J., Gill, H., Smart, J. Gopal, P.K. (). Selection and characterization of Lactobacillus and Bifidobacterium strains for use as probiotics. *Int. Dairy J.* 1998; 8: 993–1002.

Priest, F.G. Extracellular enzyme synthesis in the genus Bacillus. *Bact. Rev.* 1977; 41: 711–753.

Pritchard, G., Coolbear, T. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. *FEMS Microbiol. Rev.* 1993; 12: 179-206.

Raa, J., Gildberg, A. Fish silage: A review. Crit. Rev. Food Sci. Nutr. 1982; 16: 383-419.

Raa, J., Gildberg, A., Strom, T. Silage production: Theory and practice. In: D.A. Ledward, A.J. Taylor, R.A. Lawrie (Eds) *Upgrading Waste for Feeds and Food.* Butterworths, London, 1983; pp. 117-132.

Rajendra, W. High performance liquid chromatographic determination of amino acids in biological samples by precolumn derivatization with O-phthdialdehyde. *J. Liquid Chromatogr.* 1987; 10: 941–955.

Rao, M.S., Munoz, J., Slevens, W.F. Critical factors in chitin production by fermentation of shrimp biowaste. *Appl. Microbiol. Biotech.* 2000; 54: 808-813.

Rattagool, P., Surachatmrongratane, S., Wongchinde, N. Fish silage in Thailand, Further trials on broilen chicken. In: Proceedings IPFC Workshop on fish silage FAO. Fishery Report. No. 230, 1980; 59-63.

Rattanakit, N., Plikomol, A., Yano, S., Wakayama, M., Tachiki, T. Utilization of shrimp shellfish waste as a substrate for solid-state cultivation of *Aspergillus* sp. S 1 - 13 : Evaluation of a culture based on chitinase formation which is necessary for chitin-assimilation. *J. Biosci. Bioeng.* 2002; 93: 550-556.

Rattanakit, N., Yano, S., Wakayama, M., Plikomol, A., Tachiki. T. Saccharification of Chitin Using Solid-State Culture of *Aspergillus* sp. S I-I 3 with Shellfish Waste as a Substrate. *J. Biosci. Bioeng.* 2003; 95: 391-396.

Ray, B. The need for food biopreservation. In: B. Ray, M. Daeschel (Eds.) *Food biopreservatives of microbial origin.* CRC Press, Boca Raton, Florida, 1992; pp. 1–23.

Richards, M., Macrae, R.M. The significance of the use of hops in regard to the biological stability of beer. The development of resistance to hop resins by strains of lactobacilli.. J. Inst. Brewing, 1964; 70: pp. 484–488.

Roberts, R.F., Zottola, E.A., Mckay, L.L. Use of a nisin producing starter culture suitable for Cheddar cheese manufacture. *J. Dairy Sci.* 1992; 75: 2353–2363.

Rockway, S.W. Absorbitol fat binding report. *Pharmanutrients* 2000; 2: 1-8.

Roig-Sagues, A. X., Hernandez-Herrero, M., Lopez-Sabater, E.I., Rodriguez-Jerez, J.J., Mora-Ventura, M.T. Histidine decarboxylase activity of bacteria isolated from raw and ripened Salchichon, a Spanish cured sausage. *J. Food Prot.* 1996; 59: 5, 516-520.

Ruiz-Barba, J.L., Cathcart, D.P., Warner, P.J., Jimenez-Diaz, R. Use of Lactobacillus plantarum LPCO10, a bacteriocin producer, as a starter culture in Spanish-style green olive fermentations. *Appl. Environ. Microb.* 1994; 60: 2059–2064.

Rutherford, F., Austin, P.R. In: Proceedings of First Int. Conf. Chitin/ Chitosan, MIT, Cambridge, 1978; 182–921.

Samuels, W.A., Fontenot, J.P., Allen, V.G., Abazinge, M.D.A. Seafood processing wastes ensiled with straw: utilization and intake by sheep. *J. Anim. Sci.* 1991; 69: 4983-4992.

Sandine, W.E. Commercial production of dairy starter cultures. In: T.M. Cogan, J.P. Accolas (Eds) *Dairy starter cultures.* Wiley-VCH, New York, 1996; pp. 191–206.

Santhosh, S., Sini, T.K., Anandan, R., Mathew, P.T. Effect of chitosan supplementation on the antitubercular drugs-induced hepatotoxicity in rats. *Toxicology* 2006; 219: 53-59.

Sarkar, P.K., Cook, P.E., Owens, J.D. (1993) *Bacillus* fermentation of soybeans. *World. J. Microb. Biot.* 1993; 9: 295-299.

Sarkar, P.K., Tamang, J.P. Changes in the microbial profile and proximate composition during natural and controlled fermentations of soybeans to produce kinema. *Food Microbiol.* 1995; 12: 317–325.

Sastry, C.S.P.,Tummuru, M. Spectrophotometric determination of tryptophan in proteins. *J. Food Sci. Tech.* 1985; 22: 146-147

Schleifer, K.H., Ehrmann, M., Beimfohr, C., Brockmann, E., Ludwig, W., Amann, R. Application of Molecular Methods for the Classification and Identification of Lactic Acid Bacteria *Int. Dairy J.* 1995; 5: 1081-1094.

Schleifer, K.H., Ludwig, W. Phylogenetic relationships of lactic acid bacteria. In: B.J.B Wood, W.P. Holzapfel *The Lactic Acid Bacteria, Vol. II: The Genera of Lactic Acid Bacteria,* Chapman and Hall, London, 1994; 123-128.

Shahidi, F., Synowiecki, J. Isolation and characterization of nutrients and value-added products from snow crab (Chinoecetes opilio) and shrimp (Pandalus borealis) processing discards. *J. Agr. Food Chem.* 1991; 39: 1527-1532.

Shimahara, K., Takiguchi, Y. Preparation of crustacean chitin. In: W.A. Wood, S.T. Kellog (Eds) *Methods in Enzymology.* Academic press, Newyork, 1988; 161: pp. 417-423.

Shirai, K. Utilisation of shrimp wastes for chitin, protein and pigments recovery by microbial route (Ph.D. Thesis). Universidad Autonoma Metropolitana, Mexico City, Mexico, 1999; 54-59

Shirai, K., Guerrero, I., Huerta, S., Saucedo, G., Gonzalez, R.O., Castillo, A., Hall,G.M. Effect of initial glucose concentration and inoculation level of lactic acid bacteria in shrimp waste ensilation. *Enzyme Microb. Tech.* 2001; 28: 446-452.

Shirai, K., Guerrero, I., Huerta, S., Saucedo, G., Rodriguez, G., Hall, G.M. Aspects in protein breakdown during the lactic acid fermentation. In: A. Domard, G.A.F. Roberts, K.M. Varum (Eds) *Advances in chitin science*, 1997; 2: Jaques Andre Publisher, Lyon, 1997; 56-63.

Silla, M.H. Biogenic amines: their importance in foods. *Int. J. Food Microbiol.* 1996; 29: 213– 231.

Silliker, J.H., Elliot, R.P., Baird-Parker, A.C., Bryan, F.L., Christian, J.H.B., Clark, D.S., Olson, J.C, Roberts, T.A. In: Microbial Ecology of Foods. AcademicPress, USA, 1980; 2: 589-592.

Simpson, B.K., Gagne, N., Simpson, M.V. Bioprocessing of chitin and chitosan. In: A.M. Martin (Ed) *Fisheries Processing*. Chapman and Hall, London, 1994; pp. 155-173.

Sini, T.K., Santhosh, S., Mathew, P.T. Study of the influence of processing parameters on the production of carboxymethylchitin. *Polymer* 2005; 46: 3128–3131.

Sini, T.K., Santhosh, S., Mathew, P.T. Study on the production of chitin and chitosan from shrimp shell by using *Bacillus subtilis* fermentation. *Carbohydr. Res.* 2007; 342: 2423-2429.

Steinkraus, K.H. African alkaline fermented foods and their relation to similar fermented foods in other parts of the world. In: A. Westby, P.J.A. Reilly (Eds) *Proceedings of a Regional Workshop on Traditional African Foods - Quality and Nutrition*, International Foundation for Science, Stockholm, 1992; pp. 87-92.

Steinkraus, K.H. Indigenous fermented foods involving an alkaline fermentation. In: K.H. Steinkraus (Ed.) *Handbook of Indigenous Fermented Foods*. Marcel Dekker, New York, USA, 1995; pp. 349–362.

Straub, B.W., Tichaczek, P.S., Kicherer, M., Hammes, W.P. Z. *Lebensm. Unters.Forsch.* 1994; 199: 9-15.

Strongin, A.Y., Stepanov, V.M. In: *Intracellular serine proteinases from spore-forming bacilli.* Translated by Plenum Publishing Corporation from Biokhimiya, 19824; 6: 1347–1363.

Sullivan, L.O, Ross, R.P., Hill, C. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie* 2002; 84: 593–604

Tabor, C.W., Tabor, H. Polyamines in microorganisms. *Microbiol. Rev.* 1985; 49: 81– 99.

Taha, S.M., Swailam, H.M. Antibacterial activity of chitosan against aeromonos hydrophila. *Nahrung* 2002; 46: 337-340.

Takeda, M., Abe, E. Isolation of crustacean chitin. Decalcification by sodium ethylene diamine tetra acetate and enzymatic hydrolysis of incidental proteins. *Noriasho Suidson Koshuro Kenkyu Hokoku* 1962; 11: 339-406.

Takeda, M., Katsura, H. Purification of king crab chitin. *Suisan Daigaku Kenkyu Hokoku* 1964; 13: 109-116.

Tamang, J.P., Tamang, B., Schillinger, U., Franz, C.M.A.P., Gores, M., Holzapfel, W.H. Identification of predominant lactic acid bacteria isolated from traditionally fermented vegetable products of the Eastern Himalayas. *Int. J. Food Microbiol.* 2005; 105: 347-356.

Taylor, S. Histamine food poisoning: toxicity and clinical aspects - a review CRC. *Crit. Rev. Toxicol.* 1986; 91-128.

ten Brink, B., Damink, C., Joosten, H.M.L.J., Huisin't Veld, J.H.J. Occurrence and formation of biologically active amines in foods. *Int. J. Food Microbiol.* 1990; 11: pp. 73-84.

ten Brink, B., Minekus, M., Van der Vossen, J.M.B.M., Leer, R.J., Huisin't Veld, J.H.J. Antimicrobial activity of lactobacilli: preliminary characterization and optimization of production of acidocin B, a novel bacteriocin produced by *Luctobacih acidophilus M46. J. Appl. Bacteriol.* 1994; 77: 140-148.

Teng, W.L., Khor, E., Tan, T.K., Lim, L.Y., Tan, S.C. Concurrent production of chitin from shrimp shells and fungi. *Carbohyd. Res.* 2001; 332: 305–316.

Teniola, O.D., Odunfa, S.A. The effects of processing methods on the levels of lysine,methionine and the general acceptability of *ogi* processed using starter cultures. *Int. J. Food Microbiol.* 2001; 63: 1–9.

Tolaimate, A., Desbrieres, J., Rhazi, M., Alagui, A. Contribution to the preparation of chitins and chitosans with controlled physico-chemical properties, *Polymer* 2003; 44: 7939-7952.

Tsigos, I., Bouriotis, V. Purification and Characterization of Chitin Deacetylase from *Colletotrichum lindemuthianum*. *J. Biol. Chem.* 1995; 270: 26286–26291.

Tsuru, D., Mc Conn, J.D., Yasunobu, K.T. Chitin and applications. *Bioch. Biophys.Res. Commun.* 1964; 15: 367-371.

Vaaje-Kolstad, G., Houston, D.R., Riemen, A.H.K., Eijsink, V.G.H., van Aalten, D.M.F. Crystal Structure and Binding Properties of the *Serratia marcescens* Chitin-binding Protein CBP21. *J. Biol. Chem.* 2005; 280: 11313–11319.

Vachova, L., Kucerova, H. Calcium as a regulator of Bacillus megaterium cytoplasmic proteolytic activity in vitro. *FEMS Microbiol. Lett.* 1998; 162: 283-288.

van Kranenburg, R., Kleerebezem, M., Vlieg, J.H., Ursing, B.M., Boekhorst, J., Smit, B.A., Ayad, E.H.E., Smit, G., Siezen, R.J. Flavour formation from amino acids by lactic acid bacteria: predictions from genome sequence analysis. *Int. Dairy J.* 2002; 12: 111–121.

Van W.H.J, Heydenrych, C.M.S. The production of naturally fermented fish silage using various lactobacilli and different carbohydrate sources. *J. Sci. Food Agr.* 1985; 36: 1093–1103.

Venugopal, V., Alur, M.D., Nerkar, D.P. Solubilization of fish proteins using immobilized microbial cells. *Biotechnol. Bioeng.* 1989; 33: 1098 –1103.

Vereecken, K.M., Van Impe, J.F. Analysis and practical implementation of a model for combined growth and metabolite production of lactic acid bacteria. *Int. J. Food Microbiol.* 2002; 73: 239– 250

Vinderola, C.G., Reinheimer, J.A. Lactic acid starter and probiotic bacteria: a comparative in vitro study of probiotic characteristics and biological barrier resistance. *Food Res. Int.* 2003; 36: 895-904.

Visser, S. Proteolytic enzymes and their relation to cheese ripening and flavor: An overview. *J. Dairy Sci.* 1993; 76: 329-350.

Vogel, R.F., Pohle, B.S., Tichaczek, P.S., Hammes, W.P. The competitive advantage of Lactobacillus curvatus LTH 1174 in sausage fermentations is caused by formation of curvacin A. *Syst. Appl. Microbiol.* 1993; 16: 457–462.

Wang, Q., Yamabe, K., Narita, J., Morishita, M., Ohsumi, Y., Kusano, K., Shirai, Y., Ogawa, H.I. Suppression of growth of putrefactive and food poisoning bacteria by lactic acid fermentation of kitchen waste. *Process Biochem.* 2001; 37: 351–357.

Wang, S.L, Chio, S.H. Deproteinization of shrimp and crab shell with the protease of *Pseudomonas aeruginosa* K-187. *Enzyme Microb. Tech.* 1998; 22: 629–633.

Wang, S.L., Chio, S.H., Chang, W.T. Production of chitinase from shellfish wastes by *Pseudomonas aeruginosa* K-187. *Proc.Natl. Sci. Counc. ROC (B)* 1997; 21: 71–79.

Weerkamp, A.H., Klijn, N., Neeter, R., Smit, G. Properties of mesophilic lactic acid bacteria from raw milk and naturally fermented raw milk products. *Neth. Milk Dairy J.* 1996; 50: 319-332.

Weinberg, Z.G., Muck, R.E. New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiol. Rev.* 1996; 19: 53-68

Whittenbury, R. An investigation of the lactic acid bacteria. PhD. Thesis. Edinburgh University. U.K., 1961; 56-62.

Wijtzes, T., Bruggeman, M.R., Nout, M.J.R., Zwietering, M.H. A computerised system for the identification of lactic acid bacteria Short communication. *Int. J. Food Microbiol.* 1997; 38: 65–70.

Wiseman, J. Green, S., Cole, D.J.A. Nutritive value of oily-fish silage when used to replace ingredients of plant origin in growing pig diets. *Proc. Bri. Soc. Anim. Prod.* 1982; 3: 356-361.

Wood, B.J.B. In: *Microbiology of fermented foods.* Blackie Academic & Professional, London, 1997; 23-28.

Wood, B.J.B., Holzapfel, W.H. *The genera of lactic acid bacteria.* Blackie Academic & Professional, London, 1995; 78-81.

Yang, J.K., Shih, I.L., Tzeng, Y.M., Wang, S.L. Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme Microb. Tech.* 2000; 26: 406–413.

Zakaria, Z., Hall, G.M., Shena, G. Lactic acid fermentation of scampi waste in a rotating horizontal bioreactor for chitin recovery. *Process Biochem.* 1998; 33: 1-6.

LIST OF PUBLICATIONS

- Study on the production of chitin and chitosan from shrimp shell by using *Bacillus subtilis* fermentation. ***Carbohydrate Research***. Elsevier, U.S.A., 2007; 342: 2423-2429.

- Effect of different organic acids in silage preparation from shrimp shell waste. ***Fishery Technology***, SOFTI, India, 2007; 44: 43-48.

- Study of the influence of processing parameters on the production of carboxymethylchitin. ***Polymer***, Elsevier, U.K., 2005; 46: 3128-3131

- Effect of chitosan supplementation on antitubercular drugs-induced hepatotoxicity in rats. ***Toxicology*** (Elsevier, Ireland), 2006; 219: 53-59.

- Biochemical studies on the antiulcer effect of glucosamine on antioxidant defense status in experimentally induced peptic ulcer in rats. ***Journal of Clinical Biochemistry and Nutrition*** (Academic Publishers, Japan) 2005; 37: 61-66.

- Protective effect of glucosamine against ibuprofen-induced peptic ulcer in rats. ***Journal of Gasteroenterology and Hepatology*** (Blackwell synergy, Australia) 2007; 22: 949–953.

- Hepatoprotective activity of chitosan against isoniazid and rifampicin-induced toxicity in experimental rats. ***European Journal of Pharmacology*** (Elsevier, France) 2007; 572: 69-73.

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Carbohydrate RESEARCH

udy on the production of chitin and chitosan from shrimp shell by using *Bacillus subtilis* fermentation

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t—Fermentation of shrimp shell in jaggery broth using *Bacillus subtilis* for the production of chitin and chitosan was inves- It was found that *B. subtilis* produced sufficient quantities of acid to remove the minerals from the shell and to prevent spoil- anisms. The protease enzyme in *Bacillus* species was responsible for the deprotenisation of the shell. The pH, proteolytic , extent of demineralization and deprotenisation were studied during fermentation. About 84% of the protein and 72% of erals were removed from the shrimp shell after fermentation. Mild acid and alkali treatments were given to produce char- ic chitin and their concentrations were standardized. Chitin was converted to chitosan by N-deacetylation and the properties n and chitosan were studied. FTIR spectral analysis of chitin and chitosan prepared by the process was carried out and com- ith spectra of commercially available samples.

: *Bacillus subtilis*; *Metapenaeopsis dobsoni*; Shrimp shell; Chitin; Chitosan

1. Introduction

, the second most abundant biopolymer next to se and its derivatives like chitosan, carboxymethyl , etc., are widely recognized to have immense ations in many fields.[1] They are widely used in od industry, medicinal fields, chemical industries, , water treatment plants, etc.[2,3] Glucosamine is r value-added product prepared from chitin by lysis and it has versatile applications in pharma- .[4] The prerequisites for the greater use of these lymers in various industries are cost of the manu- ing process and the technical grade with specific rties.[5] The commercial method of preparation of from shrimp shell involves strong acid and alkali ent to remove the minerals and proteins, respec- .[6] However, the use of these chemicals causes depo- isation of the product and therefore affects rties such as molecular weight, viscosity and de- gree of deacetylation. These chemical treatment methods bring about hazardous environmental problems like disposal of wastewater. The cost of the chemicals is another drawback of this approach.

To overcome the problems of chemical treatments, different microorganisms[7–9] and proteolytic enzymes[10,11] were used to remove the proteins and mineral content. During fermentation with microbes, deprotenisation takes place by the activity of proteases in the micro-organisms and demineralization by the acid produced by the microorganisms during fermentation.[12]

Commercial bacterial proteases are derived from *Bacillus* broth.[13] *B. subtilis*, the commercial starting material, contains a neutral protease, that is, characterized by its pH activity and neutral stability.[14] Zinc is essential for catalytic activity and calcium is required to maintain the structural rigidity of the enzyme. Many reports have demonstrated the application of proteases for the degradation of proteinaceous waste to useful biomass.[15–17] *B. subtilis* and *B. firmus* are the most common exploited species. Many authors[14,18] reported the application of *B. subtilis* for deprotenisation of crustacean waste to produce chitin.

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In the present work, an attempt was made to produce tin by fermentation using *B. subtilis* and to study its ious properties. Chitosan was also prepared from chitin and its properties were analyzed.

2. Results and discussions

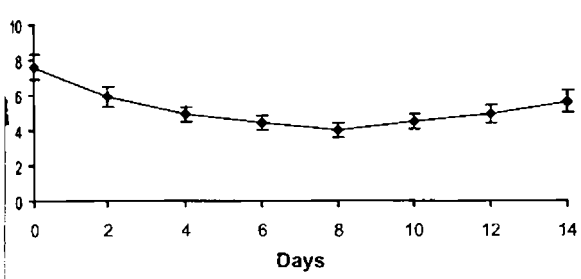
Changes in pH

ring fermentation, the pH of the liquor samples first owed a decreasing trend up to the eighth day and reafter started rising (Fig. 1). An initial decrease in was noticed in samples and that could be due to ability of the strain *B. subtilis* to initially use sugar substrates for their growth[19] and simultaneously they duce acid via pyruvate.[20] Low pH condition prevents decay of shrimp shell, which are colonized by spoil- and pathogenic organisms.[21] By the eighth day, out 87% of the sugar in the jaggery broth was utilized the organism. Therefore, when the sugar substrate s depleted, acid production decreased and pH started reasing.[22] The acid produced was responsible for the nt of demineralization noticed in the samples at the of fermentation.[12] During fermentation, proteolysis urs and ammonia is released due to utilization of ino acids for the growth of the bacteria.[19,23,26] This might have caused the increase in pH.

Extent of deprotenisation and demineralisation

deprotenisation of the shrimp shells was brought out by the activity of the neutral protease of *B. sub-*.[13,18] Yang et al.[14] found that the optimum activity the protease produced by *Bacillus subtilis* took place 30 °C and pH of 6. In the present work also the pro- se showed maximum proteolytic activity of 10.6875 g rosine/g protein after 48 h of fermentation when the of the liquor was 5.9. The proteolytic activity re- ined fairly constant for 5–6 days during fermentation ig. 2). These changes are clearly supported by the orts of Allagheny et al.[26] and Sarkar et al.[19]

During fermentation, when the available carbo- drate source was utilized by the microorganisms for ir growth, acid was released and this was responsible



re 1. Changes in pH of the protein liquor.

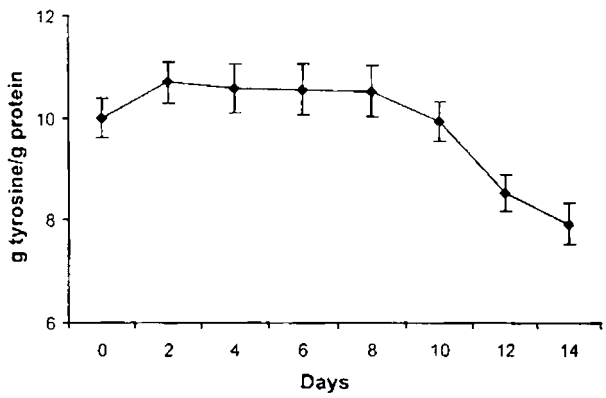


Figure 2. Changes in proteolytic activity.

for calcium solubilization from the shrimp shell.[12] As the calcium ion concentration increased in the fermenting media, proteolytic activity showed a decreasing trend. Tsuru et al.[27] reported that calcium is recognized to prevent protease autolysis.

2.3. Chemical treatment of fermented residue

Table 1 shows the protein and ash content of the fermented residue. About 84% of the protein and 72% of the minerals were removed from the shrimp shell at the end of fermentation. To produce chitin of standard quality, residual protein and ash content of the fermented residue have to be removed by mild acid and alkali treatments. Different concentrations of hydrochloric acid and sodium hydroxide were tried to remove ash and protein, respectively (Figs. 3 and 4). Considering the statistical significance ($P < 0.05$) in the extent of demineralization trials, we have used 0.8 N HCl for the demineralization study. Similarly, among different alkali concentrations tried, 0.6 M NaOH was used for the deprotenisation ($P < 0.05$).

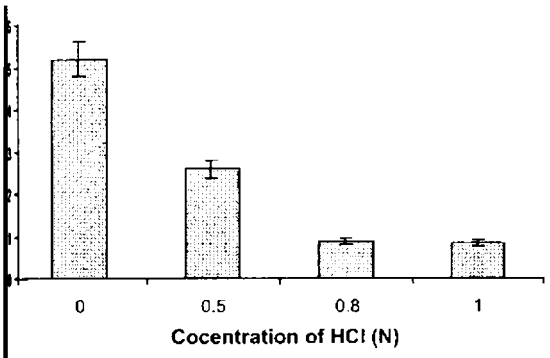
Many previous papers reported the chemical treatment of the fermented residue to remove the residual protein and ash.[8,28,29] The optimum concentrations of the acid and alkali required depend on the residual protein and ash content. This was dependent on the type of the raw material, type of the starter culture, fermentation time, etc.

2.4. Characteristics of chitin prepared from shrimp shell

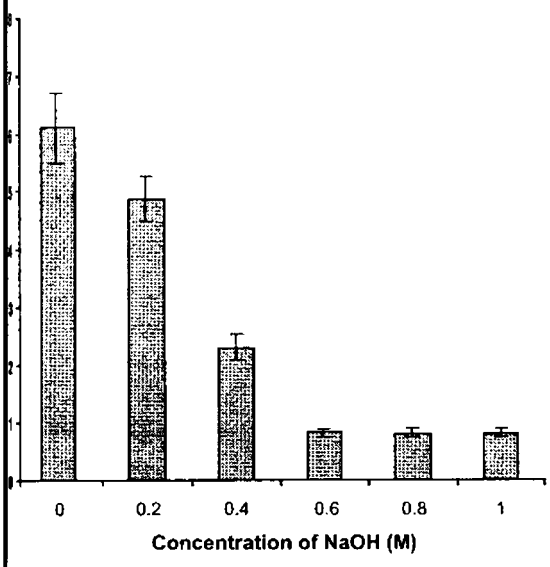
Table 2 presents the characteristics of the chitin prepared by this treatment. The chitin obtained is α-chitin

Table 1. Protein and ash content of the fermented residue

Particulars	% of protein content	% of ash content
Raw material	38.10 ± 2.15	18.538 ± 0.89
Fermented residue	6.096 ± 0.4	5.191 ± 0.3



3. Percentage of ash content in the fermented residue treated ifferent concentrations of acid.



4. Percentage of protein content in fermented residue treated ifferent concentrations of alkali.

2. Properties of chitin prepared

isture	5.1 ± 0.4%
h	0.854 ± 0.04%
tein	0.815 ± 0.5%
tin	93.2 ± 0.6%
earance	White flakes
N-acetylation	84.4 ± 5.3
	7.8 ± 0.2

the chitin content of the product was found to be %. Mineral content (calcium, sodium, potassium magnesium) in the chitin prepared from the shrimp is shown in Figure 5. From the figure, it is clear calcium content is more than other minerals in chi- This is due to the fact that the main constituent of rals in shrimp shell is calcium carbonate.[22] Mild treatment after fermentation, reduced the mineral ent to permissible limits in the chitin. One of the fac-

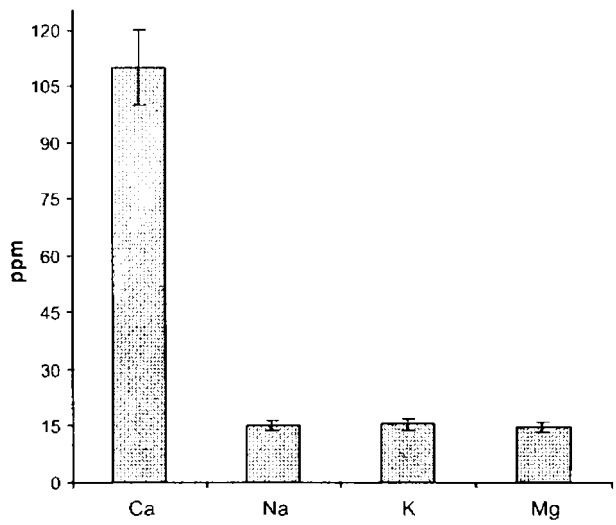


Figure 5. Mineral content of the chitin.

tors determining the good quality of chitin is the low mineral content.[30]

The FTIR spectrum of the chitin prepared by this method is shown in Figure 6. The spectrum was found to be similar to that of the commercial chitin (Fig. 7). There are no impurities found. Because, it has a better percentage of individual functional groups, the chitin prepared by the *Bacillus* fermentation compares favorably with the commercial chitin, with regard to purity. The percentage of N-acetylation, calculated from the FTIR spectrum, was remarkable, 84.4%. Other characteristics of the chitosan prepared are given in Table 3. The FTIR spectrum of the prepared chitosan is presented in Figure 8 and is similar to that of commercial chitosan (Fig. 9).

3. Conclusion

From this study it was found that *B. subtilis* was found to be an efficient starter culture for fermentation of shrimp shells. About 84% of the protein and 72% of the minerals were removed from the fermented residue at the end of fermentation. Chitin and chitosan were prepared from the fermented residue and the physiochemical properties of these products were found to be similar to commercial grades of these materials.

4. Materials and methods

4.1. Shrimp shell

The shrimp (*Metapenaeopsis dobsoni*) shells used for the experiment were procured in fresh condition from the shrimp processing plant located at Cochin, Kerala,

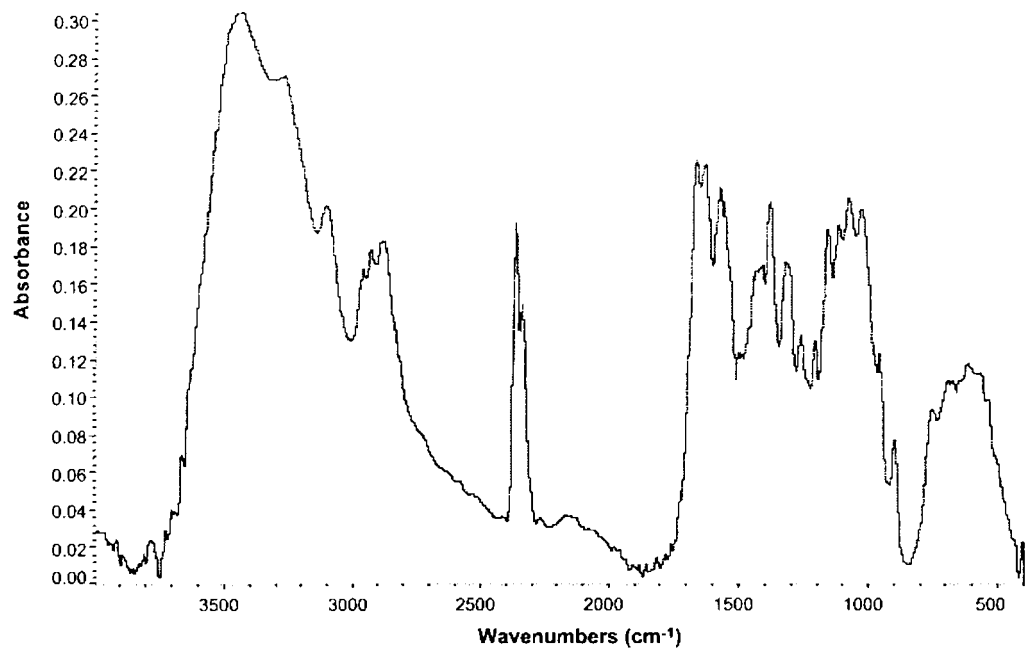


Figure 6. FTIR spectrum of chitin prepared by the *Bacillus* fermentation.

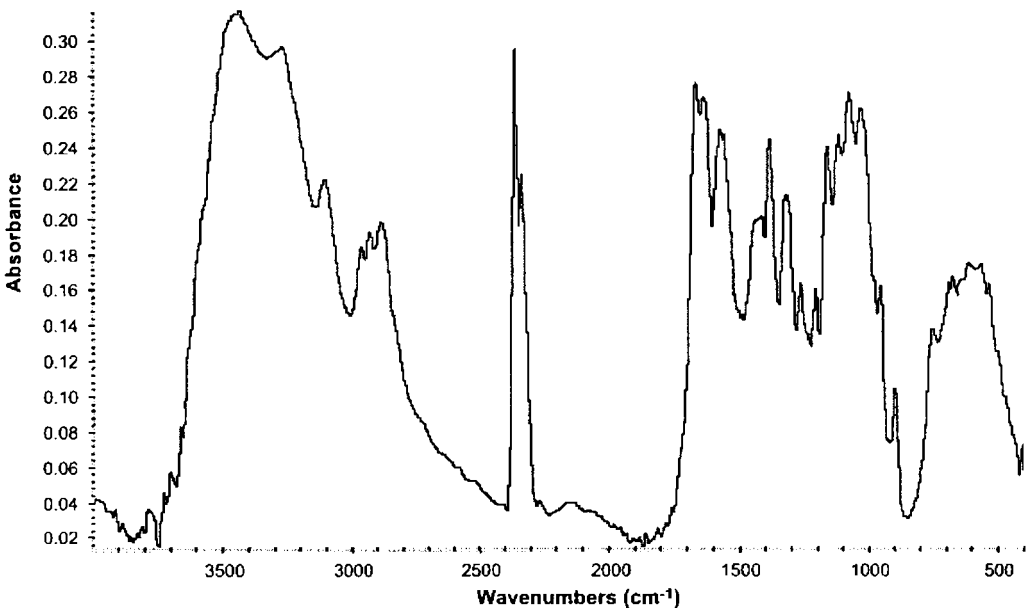


Figure 7. FTIR spectrum of commercially available chitin.

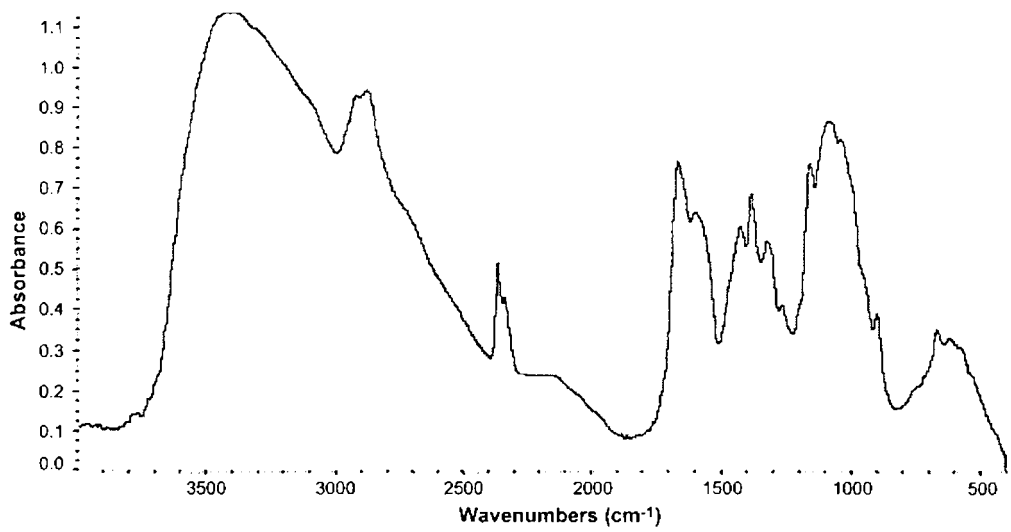
Table 3. Characteristics of chitosan prepared

Moisture (%)	7.6 ± 0.5
Ash (%)	0.832 ± 0.06
Appearance	Off white flakes
Acid insoluble ash	Nil
Solubility (1% of chitosan solution) in 1% acetic acid	95%
Viscosity (m Pa S)	1680 + 138
Molecular weight ($\times 10^5$) (Da)	2.56 ± 0.15
Degree of deacetylation (%)	81 ± 7

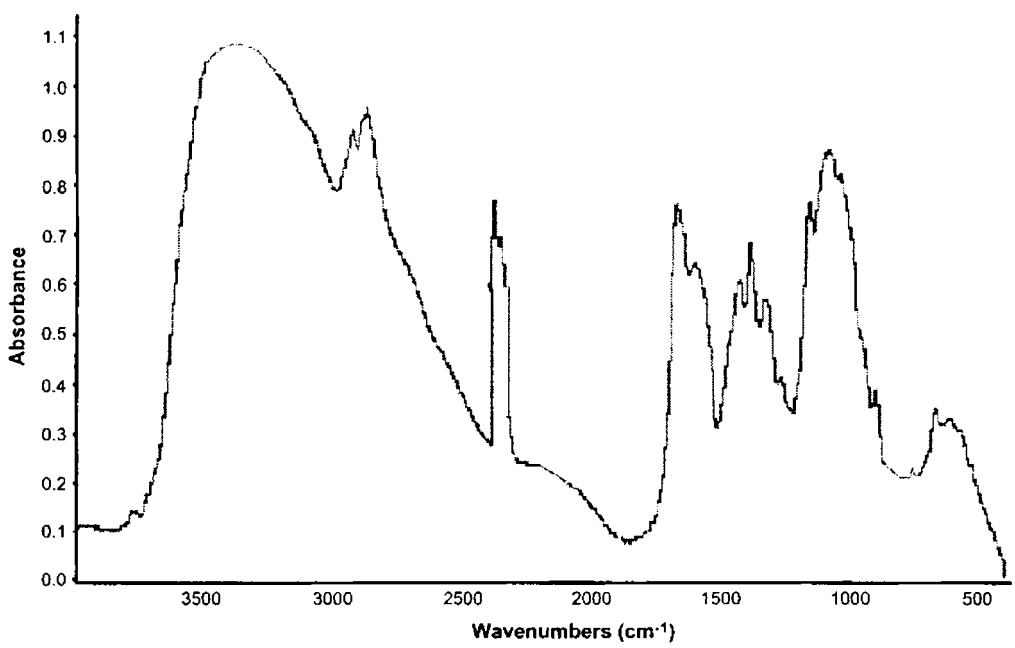
India. The shell waste brought to the laboratory was minced in a meat mincer and used for the study.

4.2. Jaggery

Jaggery was used for the preparation of media for *Bacillus* growth and was obtained from the local market at Cochin. Its sucrose content was estimated and about



8. FTIR spectrum of chitosan.



9. FTIR spectrum of commercial chitosan.

w/w broth prepared for the study was sterilized at i for 15 min.

Inoculum preparation

eze-dried culture of *B. subtilis* (ACC No. *121) was ned from the Institute of Microbial Technology ECH), Chandigarh, India. The *B. subtilis* strain maintained in nutrient agar slopes at 4 °C. The ilum was prepared by adding a loop full of cells)mL nutrient broth and incubated at 30 °C for Then, 0.5 mL from this inoculum was transferred to 100 mL of sterilized 20% w/v jaggery broth and incubated at 30 °C for 24 h. The prepared inoculum yielded a cell concentration of approximately 10^8 CFU/mL.

4.4. Fermentation

Thoroughly minced shrimp waste (200 g) was mixed with 24 h incubated 20% w/w jaggery broth (200 mL) containing 10^8 CFU/mL of *B. subtilis*. The flask was tightly closed and kept for fermentation for 15 days. Liquor samples were taken at 48 h intervals and analyzed for pH, proteolytic activity and sugar concentration.

ter the completion of fermentation, sediments were moved, washed and estimated for its ash, chitin and otein contents.

Preparation of chitin

id fractions obtained after fermentation were treated h different concentrations of HCl (0.2, 0.5, 0.8 and M) in 1:15 (solid:acid) ratio for 2 h at room tempera- e (30 ± 2 °C). The samples were then washed well d treated with different concentrations of NaOH 2, 0.4, 0.6, 0.8, 1 and 1.2 M) in 1:15 (solid:alkali) ratio deprotenisation. Protein, ash and chitin contents e estimated. The FTIR spectra of the chitin samples re also measured and compared with that of the com- rcial chitin.

Preparation of chitosan

e purified chitin was boiled with 40% (w/w) NaOH til it gets deacetylated to chitosan. Chitosan forma- n was tested by its solubility in 1% acetic acid solu- n. Moisture, ash, acid insoluble ash, viscosity, lecular weight and degree of deacetylation were mea- ed. The FTIR spectra of the chitosan samples were o measured and they are compared with the spectrum commercially available chitosan. Commercial chitin d chitosan were obtained from M/s India Sea foods d., Cochin, Kerala, India.

Analytical methods

H was measured using Cyberscan pH meter.[31] Sugar ntent was estimated by the method of Hodge and freiter.[32] The amount of protein was measured by e method of Lowry et al.[33] Proteolytic activity was alyzed with tyrosine as the standard compound.[7] Vis- sity was measured using Brookfield DV-E Viscometer th ULA spindle (SMC—0.64) at 100 rpm. Molecular ight was determined by the method of Rutherford d Austin[34] and degree of deacetylation as per the pro- dure of Muzzarelli and Rocchetty.[35] The FTIR spec- al analysis was carried out with a Nicolet AVATAR 0ESP FTIR Spectrometer. The average number of ans taken per sample was 16 in the spectral region be- een 400 and 4000 cm^{-1}. The degree of N-acetylation the samples was calculated using the following uation:

$$\%\text{N-acetylation} = (A1655/A3450) \times 115.^{36}$$

Calcium, sodium, potassium and magnesium were alyzed for chitin using Varian SpectrAA 220 Atomic bsorption Spectrometer. Moisture, ash and acid in- luble ash contents were also estimated for chitin and itosan samples as per AOAC.[37] Total nitrogen and itin nitrogen were estimated by the Kjeldahl method. Corrected protein was obtained by subtracting CN from TN and multiplying by 6.25, the Kjeldahl conversion factor for meat protein, assuming that protein has 16% nitrogen.[38]

Statistical analysis

Results were expressed as mean ± S.D. Univariate analysis of variance was carried out and the statistical comparisons were done with Duncan's test using a statistical package program (SPSS 10.0 for Windows).

References

1. Sini, T. K.; Santhosh, S.; Mathew, P. T. *Polymer* **2005**, *46*, 3128–3131.
2. Santhosh, S.; Sini, T. K.; Anandan, R.; Mathew, P. T. *Toxicology* **2006**, *219*, 53–59.
3. Muzzarelli, R. A. A. In *Advances in Chitin Science*; Jacque Andre: France, 1996; Vol. 1, pp 448–452.
4. Santhosh, S.; Anandan, R.; Sini, T. K.; Mathew, P. T. *J. Gastroen. Hepatol.* **2007**, *22*, 949–953.
5. Bustos, R. O.; Healy, M. G. *Chitin World*; Wirtschaftsverlag NW-Verlag fur neue Wissenschaft Gmbh: Bremerhaven, 1994; pp 15–25.
6. Bautista, J.; Jover, M.; Gutierrez, J. F.; Corpas, R.; Cremades, O.; Fontiveros, E.; Iglesias, F.; Vega *J. Process Biochem.* **2001**, *37*, 229–234.
7. Wang, S. L.; Chio, S. H. *Enzyme Microb. Technol.* **1998**, *22*, 629–633.
8. Cira, L. A.; Huerta, S.; Hall, G. M.; Shirai, K. *Process Biochem.* **2002**, *37*, 1359–1366.
9. Shirai, K.; Guerrero, I.; Huerta, S.; Saucedo, G.; Gonzalez, R. O.; Castillo, A.; Hall, G. M. *Enzyme Microb. Technol.* **2001**, *28*, 446–452.
10. Gagne, N.; Simpson, B. K. *Food Biotechnol.* **1993**, *7*, 253–263.
11. Broussignac, P. *Chim. Ind. Genie Chim.* **1968**, *99*, 1241–1247.
12. Rao, M. S.; Munoz, J.; Stevens, W. F. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 808–813.
13. Lauer, I.; Bonnewitz, B.; Meunier, A.; Beverini, M. *J. Chromatogr. B* **2000**, *737*, 277–284.
14. Yang, J. K.; Shih, I. L.; Tzeng, Y. M.; Wang, S. L. *Enzyme Microb. Technol.* **2000**, *26*, 406–413.
15. Wang, S. L.; Chio, S. H.; Chang, W. T. *Proc. Natl. Sci. Counc. ROC (B)* **1997**, *21*, 71–78.
16. Brine, C. J.; Austin, P. R. *Comp. Biochem. Physiol. B: Biochem* **1981**, *69*, 283–286.
17. Bustos, R. O.; Michael, H.. *Microbial Deprotenisation of Waste Prawn Shell.* In *Institution of Chemical Engineers Symposium Series*; Institution of Chemical Engineers: Rugby, England, 1994, pp 13–15.
18. Bustos, R. O.; Healy, M. G. *Biotechnology.* **1994**, *11*, 15–25.
19. Sarkar, P. K.; Cook, P. E.; Owens, J. D. *World J. Microbiol. Biotechnol.* **1993**, *9*, 295–299.
20. Leroy, F.; De Vuyst, L. *Trends Food Sci. Technol.* **2004**, *15*, 67–78.
21. Zakaria, Z.; Hall, G. M.; Shena, G. *Process Biochem.* **1998**, *33*, 1–6.
22. Legarreta, G. I.; Zakaria, Z.; Hall, G. M. In *Advances in Chitin Science*; Jacques Andre: Lyon, France, 1996; Vol. 1, pp 399–402.

Kiers, J. L.; Van laeken, A. E. A.; Rombouts, F. M.; Nout, M. J. R. *Int. J. Food Microbiol.* **2000**, *60*, 163–169.
Ohta, T. In *Legume-based Fermented Foods*; Reddy, N. R., Pierson, M. D., Salunkhe, D. K., Eds.; CRC Press: Boca Raton, 1986; pp 85–93.
Steinkraus, K. H. In *Proceedings of a Regional Workshop on Traditional African Foods—Quality and Nutrition*; Westby, A., Reilly, P. J. A., Eds.; International Foundation for Science: Stockholm, 1992; pp 87–92.
Allagheny, N.; Obanu, Z. A.; Campbell Platt, G.; Owens, J. D. *Int. J. Food. Microbiol.* **1996**, *29*, 321–333.
Tsuru, D.; McConn. J. D.; Yasunobu, K. T. *Biochem. Biophys. Res. Commun.* **1964**, *15*, 367.
Shahidi, F.; Synowiecki, J. *J. Agric. Food Chem.* **1991**, *39*, 1527–1532.
No, H. K.; Meyers, S. P.; Lee, K. S. *J. Agric. Food Chem.* **1989**, *37*, 575–579.
Tolaimate, A.; Desbrieres, J.; Rhazi, M.; Alagui, A. *Polymer* **2003**, *44*, 7939–7952.
31. Adams, M. R.; Cooke, R. D.; Twiddy, D. R. *Int. J. Food Sci. Technol.* **1987**, *22*, 105–144.
32. Hodge, J. E.; Hofreiter, B. T.. In *Methods in Carbohydrate Chemistry*; Whistler, R. L., Wolfram, M. L., Eds.; Academic Press: New York, 1962; Vol. 1, pp 380–394.
33. Lowry, O. H.; Rosenberg, N. J.; Far, A. L.; Randall, J. *J. Biol. Chem.* **1951**, *193*, 265–275.
34. Rutherford, F.; Austin, P. R. In *Proceedings of the First International Conference on Chitin Chitosan*; MIT: Cambridge, MA, 1978; pp 182–192.
35. Muzzarelli, R. A. A.; Rocchetty, R. *Annl. Chem. Acta.* **1973**, *64*, 371–379.
36. Teng, W. L.; Khor, E.; Tan, T. K.; Lim, L. Y.; Tan, S. C. *Carbohydr. Res.* **2001**, *332*, 305–316.
37. AOAC. In *Official Methods of Analysis*, 12th ed.; Association of Official Analytical Chemists: Washington, DC, 1975.
38. Adler-Nissen, J. *Enzymic Hydrolysis of Food Proteins*; Elsevier Applied Science: London, 1986, pp 135–138.

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Study of the influence of processing parameters on the production of carboxymethylchitin

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bstract

Carboxymethylchitin was prepared at different reaction temperatures and from alkali chitin with different concentrations of alkali. roperties of the product were studied. Alkali chitin were prepared using freshly prepared sodium hydroxide of 45, 50, 55, 60 and 65% (w/w) ncentration and carboxymethylated using monochloroacetic acid at controlled (35–40 °C) and uncontrolled (30–80 °C) temperature nditions. Molecular weight, viscosity, degree of deacetylation, etc. of the resultant product, i.e. carboxymethylchitin were determined. It as found that the reaction temperature has a profound influence on the property of the product than alkali concentration. A hygroscopic and mpletely water-soluble product was formed. Optimum conditions for the production of carboxymethylchitin were found to be 60% NaOH ncentration and at 35–40 °C reaction temperature. At these conditions, it was obtained with a molecular weight of 4.11×10^6 Da, viscosity 26 cP and degree of deacetylation 45.02%.

ywords: Chitin; Carboxymethylchitin; *Parapenaeopsis stylifera*

Introduction

Aquatic organisms like shrimp, crab, etc. contribute a ry good participation in our foods. Processing of these rganisms gives head, shell, etc as waste materials. They ill be easily spoiled and arises threats to the public health. onversion of waste materials into useful products is one of e solutions of environmental pollution, nowadays. hrimp shell contains 8–10% chitin [7], which is the most undant biopolymer after cellulose [6]. So, shrimp shell an be converted to chitin, a nitrogen containing olysaccharide.

Polysaccharides and their derivatives hold a major part in ur lives as medicines, cosmetics, textiles, paper, food and her branches of industry because of their unique nature in roperties such as low toxicity, biocompatibility, hydrophobicity, etc. [12]. In the midst of these polysaccharides, people show growing interest in chitin, since, it is widely distributed in nature [8,9]. The most commonly prepared derivative of chitin is chitosan by partial deacetylation of chitin [8]. The importance of chitin and chitosan has grown partly because they are renewable and biodegradable source of materials and partly because of the recent increased applications in biology, biotechnology and medicine. Carboxymethylchitin is another value shot derivative of chitin. The conversion of chitin into carboxymethylchitin came into practice by carboxymethylation reaction [2,9,13].

Carboxymethylchitin has successfully proven its use in the field of cosmetics as moisturizer, smoothener, cell activator and a cleaner for face skin conditioning. Carboxymethylchitin is widely used in food products also. Another innovative use of Carboxymethylchitin is in wound dressing, due to its hydrophobicity.

When, molecular characteristics of carboxymethylchitin are concerned the information available is very poor [3,5]. Here, comes the importance of the present work. This effort puts light on the preparation of carboxymethylchitin at different alkali concentrations and temperatures and the study of its properties.

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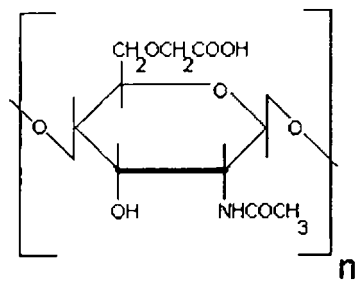


Fig. 1. Structure of carboxymethylated monomer.

Experimental

Raw material—*Parapenaeopsis stylifera* procured from cessing plant near Cochin, Kerala, India.
All chemicals were of analytical grade and were obtained n BDH (India).
Dialysis membrane obtained from Sigma chemicals (), which can retain material of molecular weight 000 or greater. Capacity—640 ml/feet.
Freeze drier-Heto drywinner(Denmark).

Preparation of chitin [10]

The fresh shell waste was washed in water and heated to ling with 0.5% aqueous NaOH in the ratio of 2:3 for min. Alkali was drained off and the shell was washed free alkali. The residue was demineralised by immersing in 1N HCl. at room temperature for 1 h with intermittent ring. The residue was filtered, washed repeatedly with er to make it acid free. Then the product, chitin obtained dried and pulverized.

Preparation of carboxymethylchitin

Carboxymethylchitin was obtained by the treatment of li chitin with monochloroacetic acid.
Fresh alkali chitin was prepared with different concentions of alkali viz. 45, 50, 55, 60 and 65% (w/w) NaOH.

1. Alkali chitin

About 10 g of chitin was mixed well with 40 ml ective alkali prepared and kept in ice for 1 h. Then, it kept overnight at −20 °C.

e 1
d of carboxymethylchitin prepared at different alkali concentrations emperature

ntage of NaOH for the aration of alkali chitin	Yield in percentage	
	Uncontrolled temperature	Controlled temperature
	36.7±1.5	37.4±1.4
	49.8±2.12	44.7±1.2
	56.5±1.7	52.0±1.6
	69.6±2.2	65.8±1.85

2.2.2. Carboxymethylchitin

To the alkali chitin prepared, poured 200 ml isopropanol and monochloroacetic acid added, at intervals, with continuous stirring. Addition of monochloracetic acid was stopped, when the whole mixture became neutral. When monochloroacetic acid was added to the mixture, reaction temperature went on increasing. For each concentration of alkali used, one set of carboxymethylation was allowed to take place at room temperature and in another set of experiment, reaction temperature maintained at 35–40 °C by using ice bath.

Resulting viscous solution was dissolved in 1 l of distilled water and then precipitated using 5 l of acetone. The precipitated mass was carboxymethylchitin and it was redissolved in distilled water. The solution was then dialysed for 24 h in running water, using a dialysis membrane, for the removal of salts and then freeze dried and stored.

Important quality parameters of carboxymethylchitin like molecular weight, degree of deacetylation, viscosity, yield, etc. were estimated. Molecular weight was determined by using Schott Gerate AVS 410 equipment [14]. By applying Schrodinger equation, degree of deacetylation was measured [11] using Spectronic Genysis spectrophotometer. Fungilab viscometer was used for viscosity determination.

3. Results and discussion

The carboxymethylchitin (Fig. 1) was prepared with alkali chitin using different concentrations of NaOH viz. 45, 50, 55, 60 and 65% (w/w). When 45, 50, 55 and 60% NaOH were used for alkali chitin preparation and when it was subjected to carboxymethylation, temperature was raised from 30 to 80 °C as more and more monochloroacetic acid was added to it and finally a neutral viscous milky solution was obtained, which was water soluble. This water-soluble product was carboxymethylchitin. Carboxymethylation was a highly exothermic reaction and so only the temperature was raised. It was found that when alkali chitin prepared with NaOH, having concentration above 60%, was used for carboxymethylation, the whole mass turned to a colloidal elastic material as the reaction proceeded, which did not show characteristic reactions of carboxymethylchitin. It implied that at higher alkali concentration of alkali chitin, carboxymethylation may not be possible.

In another set of experiment, with the same as above concentrations of alkali chitin (45, 50, 55 and 60%), reaction temperature was maintained at 35–40 °C and the reaction was slow compared to the other set, where, temperature remained uncontrolled. It was also found that carboxymethylation reaction was not possible at low temperatures below 35 °C.

Yields of carboxymethylchitin obtained at different experimental conditions were given in Table 1. Maximum

Table 2
Viscosity of 1% solution of carboxymethylchitin in water

Percentage of NaOH for the preparation of alkali chitin	Viscosity of 1% solution (cP)	
	Uncontrolled temperature	Controlled temperature
5	36±2	2114±15
0	30±1	2010±10
5	28±1	1928±7
0	22±2	1926±12

Yield was obtained when 60% NaOH was used for the preparation of alkali chitin, which was used for carboxymethylation. Under controlled temperature, carboxymethylation was very slow and the reaction would not occur completely. As a result, we got comparatively less yield at controlled temperature than uncontrolled temperature condition.

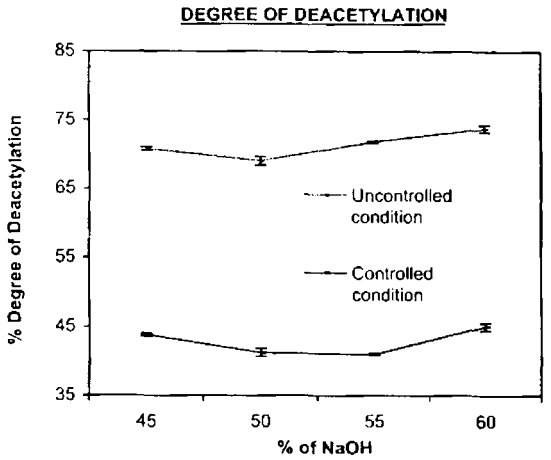
Viscosity of the samples prepared at controlled and uncontrolled temperatures using different concentrations of alkali were given in Table 2. It is well clear from the table that there was remarkable difference in viscosity of the samples prepared at controlled and uncontrolled temperatures. Thus, it was found that the temperature of the reaction had a great influence on the viscosity of the product as was in the case of the production of chitosan [8]. There was a reduction in the viscosity of the final product with increasing alkali concentration for the preparation of alkali chitin. Carboxymethylchitin with good viscosity was obtained at controlled temperature, since, the operating temperature was around 35 °C. But, at uncontrolled temperature condition, temperature was increased to a large extent and as a result thermal degradation occurred [1,] and viscosity decreased.

Molecular weight of the samples prepared was given in Table 3. Considerable difference was noticed in the case of molecular weight when the samples were prepared under controlled and uncontrolled conditions. It was found that degradation of the compound had taken place at high temperatures. Due to degradation of the product, depolymerisation of chains occurred and as a result molecular weight was decreased. The effect of alkali concentration on the molecular weight of the product under the same temperature conditions was negligible.

The degree of deacetylation of the product prepared under different conditions were shown in Graph-I. Degree of deacetylation up to 75% was reported in products obtained by carboxymethylation at uncontrolled temperature condition. But in the controlled condition, degree of deacetylation was in the range of 40–45% depending on the alkali concentration.

Table 3
Molecular weight of carboxymethylchitin prepared from alkali chitin of different alkali concentrations

Percentage of NaOH for the preparation of alkali chitin	Molecular weight ($\times 10^6$ Da)	
	Controlled temperature	Uncontrolled temperature
5	4.86±0.55	1.33±0.21
0	4.92±0.23	1.01±0.10
5	4.21±0.33	1.21±0.18
0	4.11±0.42	1.29±0.32



Graph-I. Degree of deacetylation of carboxymethylchitin.

Thus, a 65.8% yield of good quality carboxymethylchitin with high viscosity and high molecular weight was obtained when alkali chitin prepared at 60% NaOH was used for carboxymethylation and the reaction temperature was controlled at 35–40 °C. It is a hygroscopic product, which contains 10–12% moisture. From the experiment, it is well understood that the reaction temperature and alkali concentration had a great influence on its properties like viscosity, molecular weight and degree of deacetylation. Product prepared at controlled temperature condition had better properties than that obtained at uncontrolled conditions.

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References

[1] Barry JH, James NH. Polym Int 2000;49:943–8.
[2] Danilov SN, Plisko EA. J Gen Chem (Russia) 1961;31:469.
[3] Hackman RH, Goldberg M. Carbohydr Res 1974;38:35.
[4] Home HK, Foros H, Petterser H, Dornish M, Smidsrod O. Carbohydr Polym 2001;46:287–94.
[5] Inoue Y, Kaneko M, Tokura S. Rep Prog Polym Phys Jpn 1982;25: 759.
[6] Moret A, Rubio J. Miner Eng 2003;16:716.

[Legarreta GI, Zakaria Z, Hall GM. Advances in chitin science (Vol 1). France, 1996:399–406.
] Muzarelli RAA. Chitin. New York: Pergamon Press; 1977.
] Plisko EA, Nudiga LA, Danilov SN. Uspechi khimii(Progr Chem) 1977;46:1470.
[Madhavan P, Ramachandran NKG. Fish. Technol 1974;11:50–3.
[Muzarelli RAA, Rocchetty R. Annl Chem Acta 1973;64:371–9.
[12] Tombs MP, Harding SE. An Introduction to polysaccharide biotechnology. London: Taylor and Francis; 1997.
[13] Vichoreva GA, Gladyshev DY, Bazi MR, Kuznersova SY, Galbraich LS, Lavut EE, et al. Acta Polym 1991;42:330.
[14] Rutherford F, Austin PR. Proceedings of First Int Conf Chitin/Chitosan, MIT, Cambridge 1978:182–92.

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Effect of Different Organic Acids in Silage Preparation from Shrimp Shell Waste

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The comparative effect of three commonly used organic acids in the production of silage from shrimp shell waste is hereby reported. Formic acid, lactic acid and citric acid were used at 5%, 6%, and 9% concentrations respectively. Samples were kept for 16 days and at an interval of three days, changes in pH, viscosity, protein content and mineral content were analyzed. Formic acid silage had the highest liquefaction and mineral content. Amino acid profile showed highest proportion of essential amino acids in formic acid silage. In silage prepared with lactic acid and citric acid, protein content and mineral content were very low. Chitin prepared from the shell residue of formic acid silage had good quality compared to others as supported by FTIR spectrum and mineral status.

Key words : Shrimp waste, acid silage, lactic acid, formic acid, citric acid

Seafood holds a very unique position in our daily menu. But, the main curse of seafood industry is the large leaps of waste materials delivered by the processing plants. Amongst them, shrimp waste constitutes about more than one lakh tonnes per year in India (Philip & Nair, 2006). They are highly putrescible and quickly become colonized by spoilage microorganisms with the result of becoming a public health hazard (Sini *et. al.*, 2005). The best way to minimize environmental pollution is the conversion of waste materials to beneficial products. Shrimp waste contains about 10-20% calcium, 30-40% protein and 8-10% chitin (Legarreta *et. al.*, 1996). Chitin, a nitrogen containing polysaccharide, is the most valuable component in shrimp shell with numerous potential applications (Meibom *et. al.*, 2004). Chitin can be converted into chitosan, which has profound applications in various fields (Santhosh *et. al.*, 2006). Protein extracted from the shrimp shell waste has been proved to be an excellent animal feed supplement (Meyers & Benjamin, 1987).

Shrimp waste can be effectively converted into silage through an environment-friendly safe technology by using organic acids alone and in combination with *Lactobacillus* (Raa & Gildberg, 1982; Dapkevicius *et. al.*, 1998; Haard *et. al.*, 1985)

For the preparation of acid silage, choice of acid is very important. This work aims to study the effect of three most commonly used organic acids in the preparation of acid preserved shrimp silage and consequent production of chitin from the residual shell waste.

Materials and methods

Shrimp heads and shells of *Parapenaeopsis stylifera* was obtained in fresh condition from a local processing unit in Cochin, Kerala. The waste brought to the laboratory was thoroughly washed and minced in a Sumeet SP-16 electronic food preparation machine.

Formic acid, lactic acid and citric acid used were of analytical grade and procured from Sigma chemicals (USA).Potassium sorbate of

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food quality grade was obtained from SRL, Mumbai.

Silage preparation:

Formic acid silage:

About 500g of the shrimp waste was mixed well with 100ml water in glass container. To this, 30 ml (5 % of the total weight) formic acid was added gradually with constant stirring to keep the pH at 4 -4.2. It was kept for 16 days; samples were taken at intervals for analysis.

Lactic acid silage:

About 36 ml (6 % of the total weight) lactic acid was added to 500g shrimp waste, which was mixed with 100ml water and pH was maintained at 4 -4.2. 5g potassium sorbate was added as preservative. The silage thus prepared was stored for 16 days and samples were taken at intervals for analysis.

Citric acid silage:

A mixture of 500g shrimp waste and 100ml water was taken, 54 ml (9 % of the total weight) of citric acid was added with stirring and a pH in the range of 4-4.2 was maintained. 5g potassium sorbate was added for preservation.

Preparation of chitin

Shell residue obtained by the preparation of silage was converted into chitin. Residue was washed well with water and treated with 0.5 N HCl for 1 h at room temperature. Demineralised residue was washed free of acid and then boiled with 1.5 % (v/v) aqueous NaOH in the ratio of 2:3. Alkali was drained off, washed the residue with water till alkali free and dried.

Moisture, ash and crude protein were determined as per AOAC (2000). For the determination of pH values, 5ml of the sample was taken and pH was measured using Cyberscan 510 pH meter.

Viscosity of the samples was measured using Brookfield DV-E Viscometer with U1 A spindle (SMC- 0.64) at 100 rpm.

Amino acid composition was analyzed after treating the hydrolysates with 6N HCl for 24 h at 110°C as described by Moore & Stein (1963) using Shimadzu Amino acid analyzer. Tryptophan was determined colorimetrically (Fischl, 1960).

FTIR spectral analysis of the chitin prepared from shell residue of the three silages were carried out using Nicolet AVATAR 360ESP FTIR Spectrometer.

Calcium, Sodium, Potassium and Magnesium were analyzed for chitin using Varian SpectrAA 220 Atomic Absorption Spectrometer.

Results and Discussion

The proximate composition of shell waste used for silage preparation is given in Table 1.

The changes in the protein content of different silages prepared is indicated in Fig.1. It was observed that protein solubilisation was maximum during the initial 3-4 days in all the three silages and afterwards it remained almost constant. Maximum protein content was obtained in formic acid silage. About 45% protein of the raw material was liquefied in silage prepared using formic acid. But only 27% and 15% of the protein of the raw material were solubilised in lactic acid and citric acid used silages respectively.

The liquefaction of the protein is effected by means of the autolytic enzymes present in the shell. During the formation of silage, autolysis takes place and shrimp shell waste gradually liquefies as the protein matrix partially solubilizes due to its break down by endogenous enzymes (Raa & Gildberg, 1982). Since formic acid has higher pKa value (3.75) than the other two acids, it facilitates greater enzyme action and as a result more protein is leached into the silage.

Table 1. Proximate composition of shrimp shell waste

Composition	Percentage(wet weight basis)
Moisture	74.07± 2.10
Protein	11.48± 1.02
Ash	13.91±1.11
Fat	0.54±0.01

Acids used in the silage show their preservative action by the passage of

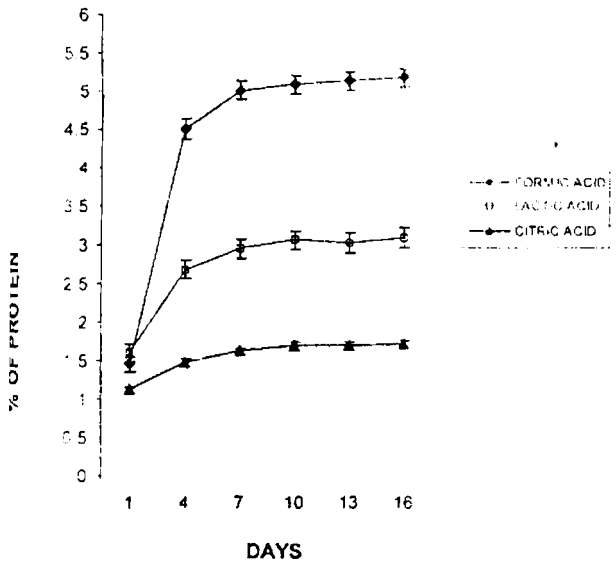

Fig. 1. Changes in protein content of the three different types of silage

undissociated acid molecule into the bacterial cell where it dissociates and lowers the pH to kill the cell (Raghunath & Gopakumar, 2002). Because of higher pKa value of formic acid, a greater proportion of the acid molecules are undissociated at higher pH and thus storage life of formic acid preserved silage is more than the other two types of silage.

To preserve the quality of the lactic acid silage and citric acid silage throughout the duration of the experiment, potassium sorbate was added to them.

Ash content of shrimp silages were very high. This was due to the high inineral content of the shrimp shell waste mainly in the form of calcium carbonate. Demineralisation of the shell waste takes place when organic acids are added to it during silage preparation. The extent of demineralization depends on the type of the organic acids and its concentration. Formic acid is stronger than lactic acid and citric acid. So, formic acid reacts strongly with the minerals, present in the shell waste, than the other two acids and as a result greater extent of demineralization was obtained by the use of formic acid. From the experiment, it was found that 75% demineralization of the shell took place when formic acid was used for silage preparation. About 72% and 67% minerals were removed from the shell with lactic acid and citric acid respectively. Lesser mineral content is more beneficial when silages are used for feed purpose. Here, citric acid silage has comparatively less mineral content than the other two silages. Changes in ash content of the silage is shown in Fig.2

Initial pH was adjusted to 4 – 4.2 in all the samples with gradual addition of respective acids. In the case of silage prepared using lactic acid, there was a reduction in pH from 4.1 to 3.67 in the first 3 days and thereafter pH remained more or less constant. This may be due

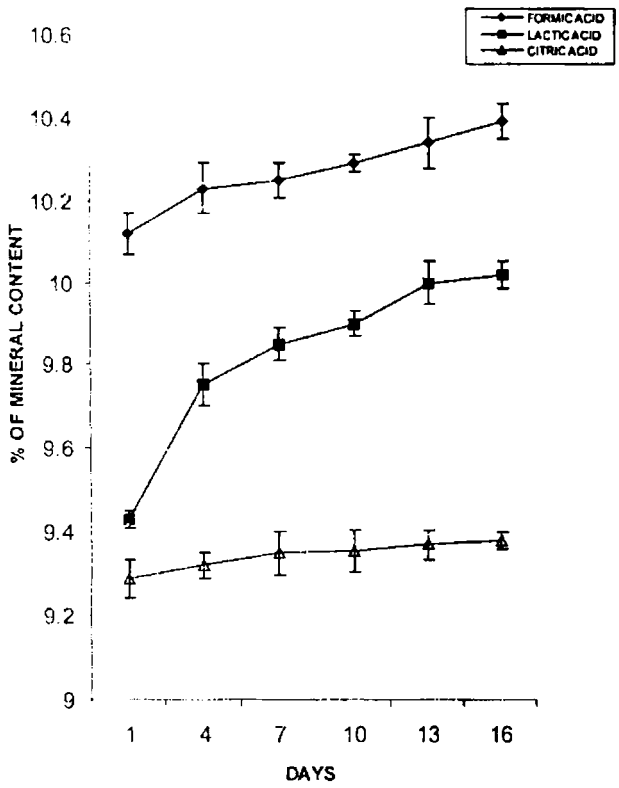

Fig. 2. Changes in mineral content in different types of silage

to the activity of the native lactic acid bacteria present in the raw shell waste. But in formic acid and citric acid treated samples, there was no significant change in pH during storage. pH changes are shown in Fig.3.

Viscosities of the samples were also measured, but it was found below 10 cp.

Amino acid compositions of the three types of silage were given in Table 2. Compared with the other two acid silages, formic acid silage has good composition of essential amino acids. Reduction in the levels of amino acid components in lactic acid silage and citric acid silage may be due to the chemical reactions between a-amino and aldehyde groups present in them (Johnson *et al.*, 1985).

Shell residue obtained after protein removal was also analyzed. The shell residue was generally found to be suitable for chitin preparation. In formic acid silage, shell waste residue had 25% ash and 55% residual protein. In lactic acid treated one, shell waste contained 28% ash and 73% protein. But in citric acid treated silage, 33% ash and 85% protein remained in the shell residue.

FTIR spectrum of chitin produced from the shell residues of the three silages are given in Figs 5a, 5b & 5c.

Chitin prepared from the shell residue of formic acid silage showed a better spectrum than that from other two acid treated residues. In lactic acid and citric acid treated chitins, the peaks (2361 cm^{-1} and 2365 cm^{-1}) responsible for hydroxyl groups, which determine the functional quality of chitin showed a gradual depletion.

Calcium, Sodium, Potassium and Magnesium were analyzed for chitin made from shell residue. Mineral composition of the chitin is given in Fig.4.

Chitin prepared with formic acid treated shell residue has less mineral content than the other two acids treated chitins. This is may be because of the high ionization value of formic acid. Chitin from citric acid has profound minerals because of the weak strength of citric acid. Lesser the mineral content, better the quality of chitin (Tolaimate *et. al.*, 2003).

Formic acid has the highest liquefaction effect and demineralization effect than lactic acid and citric acid. It has more antimicrobial activity so preservative effect is also more in the case of formic acid. More over, formic acid silage has good essential amino acid composition also. Waste residue obtained with silage can be economically utilized by its conversion into good

Table 2. Amino acid composition (g/100g crude protein) of three types of silage.

Amino acids	Formic acid silage	Lactic acid silage	Citric acid silage
Histidine	5.80	4.75	4.72
Lysine	7.18	6.96	6.98
Valine	5.72	5.56	5.49
Methionine	5.98	4.95	4.86
Leucine	7.95	7.80	6.89
Isoleucine	5.00	4.86	4.82
Phenylalanine	4.27	3.98	4.10
Arginine	2.14	1.93	1.56
Threonine	4.92	4.53	4.49
Tryptophan	0.74	0.72	0.69

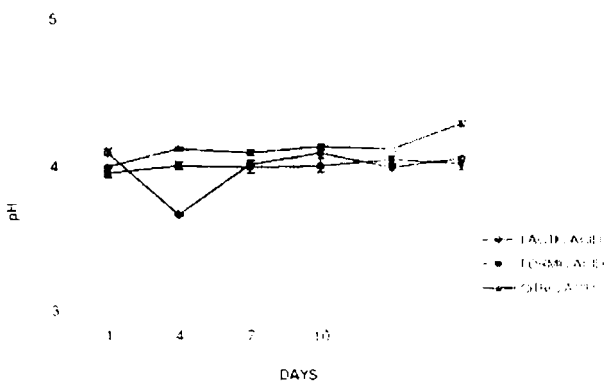

Fig. 3 Changes in pH of the three silage during storage period

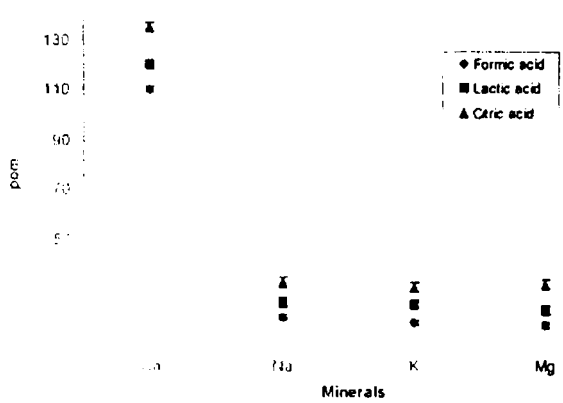

Fig. 4 Mineral composition of chitin prepared from the shell residue of different silages

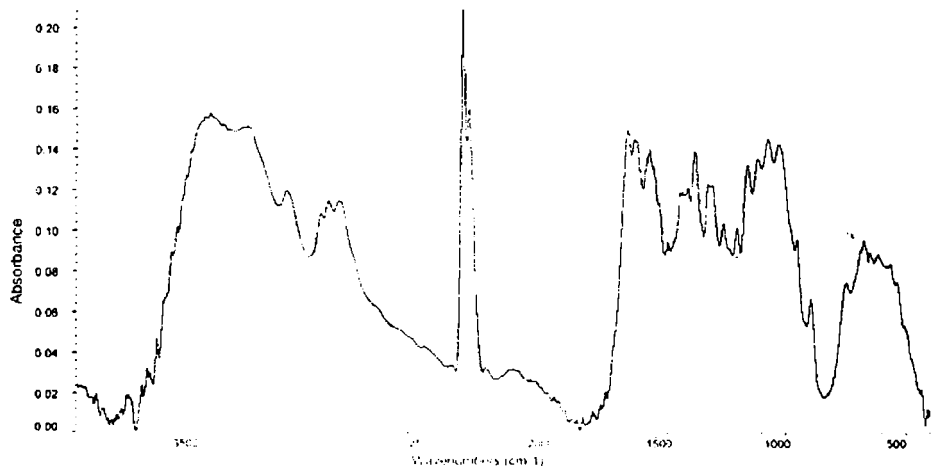

Fig. 5 a. FTIR spectrum of chitin prepared from formic acid treated shell residue

Fig. 5 b. FTIR spectrum of chitin prepared from lactic acid treated shell residue

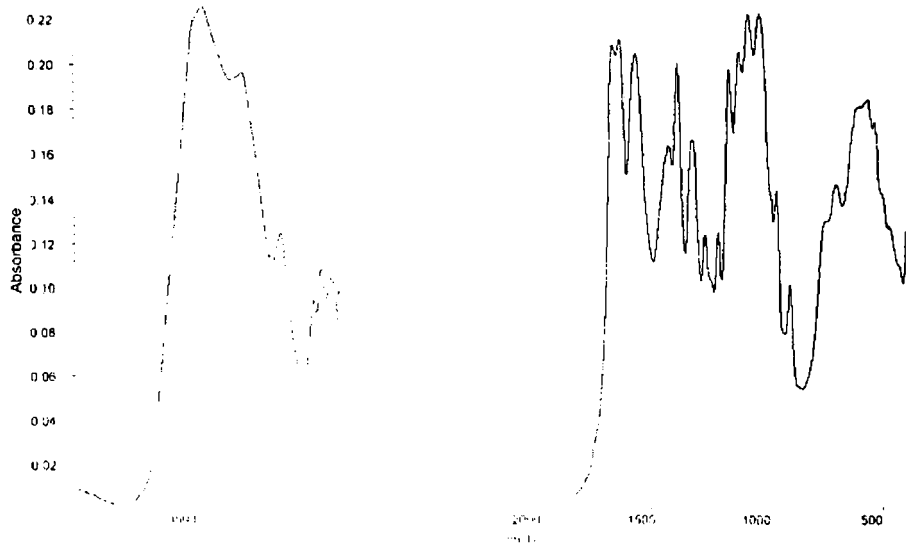

Fig. 5 c. FTIR spectrum of chitin prepared from citric acid treated shell residue

quality chitin and chitosan. Chitin prepared with the shell residue of formic acid silage has good quality as supported by FTIR spectra and mineral status.

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References

AOAC (2000). *Official Methods of Analysis*, 17th edn., Association of Official Analytical Chemists , Washington, DC, USA.

Dapkevicus, M.D.L.E., Batista, I., Nout, M.J.R., Rombouts, F.M. and Houben, J. H. (1998). Lipid and protein changes during the ensilage of blue whiting by acid and biological methods. *Food Chem.* 63, pp 97-102.

Fischl, J. (1960). Quantitative colorimetric determination of tryptophan, *J.Biol.Chem.* 235, pp 999-1001.

Haard, N.F., Kariel, N., Herzberg, G., Feltham, L.A.W., and Winter, K., (1985) Stabilization of protein and oil in fish silage for use as a ruminant feed supplement. *J. Sci. Food Agri.* 36, pp 229-241.

Johnson, R.J., Brown,N., Eason,P. and Sumner,J. (1985) The nutritional quality of two types of silage for broiler chickens. *J.Sci.Food Agric.* 36, pp 9-12

Legarreta, G.I., Zakaria, Z. and Hall, G.M. (1996). Lactic acid fermentaton of prawn waste: comparison of commercial and isolated starter culture. *Advances in Chitin Science* (Vol-I), Alan Domard, Charles Jeuniaux, Riccardo Muzzarelli and George Roberts (eds.), Jacques Andre, Lyon, France, 399p.

Lindgren, S. and Pleje, M. (1983) Silage fermentation of fish and fish waste products with lactic acid bacteria. *J. Sci. Food Agri.* 34, pp 1057-1067.

Meibom, K.L., Li, X.B., Nielsen, A.T., Wu, C.Y., Roseman, S. and Schoolnik, G.K. (2004) The *Vibrio cholerae* chitin utilization program, *Proc. Natl. Acad. Sci. USA.*,101, pp 2524-2529.

Meyers, S.P. and Benjamin, G. (1987) Feeding value of crustacean wastes can be improved through proper silage treatment. *Feedstuffs*, 6, pp 12-16.

Moore, S. and Stein, W.H. (1997) Chromatographic determination of Amino acids by use of automatic recording equipment. *Methods in Enzymology*, Sidney P. Colowick and Nathan .O. Kaplan (eds.), Academic Press, New York, USA, Vol.6. 819p.

Peter, M.G. (1995) Application and environmental aspects of chitin and chitosan. *J. Macromol. Sci. Pure. Appl. Chem.* A 32, pp 629-640.

Philip, M. and Nair, K.G.R. (2006) Ensilation of shrimp waste by *Lactobacillus fermentum*, *Fish. Technol.* 43, pp 59-64.

Raa, J. and Gildberg, A. (1982). Fish silage: A review- critical reviews in *Food Sci. Nutr.* 16, pp 383-419.

Raghunath, M.R. and Gopakumar, K. (2002). Trends in production and utilization of fish silage, *J.Food Sci. Technol.* 39, pp 103-110.

Santhosh, S., Sini, T.K., Anandan, R. and Mathew, P.T. (2006) Effect of chitosan supplementation on the antitubercular drugs-induced hepatotoxicity in rats, *Toxicology* 219, pp 53-59.

Sini, T.K., Santhosh, S. and Mathew, P.T. (2005) Study of the influence of processing parameters on the production of carboxymethylchitin, *Polymer* 46, pp 3128–3131.

Tolaimate, A., Desbrieres, J., Rhazi, M. and Alagui, A. (2003) Contribution to the preparation of chitins and chitosans with controlled physico-chemical properties, *Polymer* 44, pp 7939-7952.