CHITIN AND CHITOSAN PRODUCTIONS FROM SHRIMP SHELLS, A SEA FOOD INDUSTRIAL WASTE, BY BIOLOGICAL PROCESSES

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A DISSERTATION SUBMITTED IN PARTIAL FULLFILLMENT
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Abstract

Commercial chitin and chitosan are prepared by treating crustacean shells with chemical processes using acid and alkaline to eliminate calcium and protein from the shells. Liquid wastes derived from these chemical processes have produced high impact environmental pollution. In this study, biological processes for production of chitin and chitosan from shrimp shell waste from frozen seafood industry were investigated as potential alternative to the chemical treatment processes. Deproteinization of shrimp shells was performed by using proteolytic *Bacillus thuringiensis* SA isolated from mangrove soil in Bangkhuntien area. The protein was extracted from shrimp shells up to 90.22 ± 1.43%. Lactic acid fermentation using an effective lactic acid producing bacteria was tested for decalcification of shrimp shells. High acid producing *Lactobacillus pentosus* L7 was isolated from Nham, a Thai traditional fermented sausage, and was used for fermentation. The treated shrimp shells were decalcified and deproteinated at 97.51 ± 0.63% and 55.78 ± 4.01%, respectively. A two-step biological treatment process: lactic acid fermentation followed by deproteinization was created to improve chitin purification. Glucose was supplemented for lactic acid production of *L. pentosus* L7 in the decalcification process. When the fermentation was completed in 48 h, the calcium carbonate of the shells was solubilized for the pH of the fermenting liquid was at 3.90 ± 0.10. The amounts of residual calcium in the form of ash (1.44 ±

0.41%) and crude protein (23.24 ± 2.52%) were further eliminated by the activity of proteolytic *B. thuringiensis* SA. After decalcification and deproteinization of the shrimp shells, residual calcium and crude protein of shrimp chitin flakes were 1.72 ± 0.37% and 3.80 ± 1.28%, respectively. Chitosan was produced by deacetylation of chitin with 50% NaOH at 121°C for 5 h. The created chitosan had residual calcium, crude protein content, and degree of deacetylation of 1.58 ± 0.55%, 0.39 ± 0.26%, and 83.18 ± 1.54%, respectively. The viscosity of chitosan prepared from chitin extracted by this two-step biological process was 1,007 ± 14.73 mPa.s, whereas chitosan prepared from chitin made from the same lot of shrimp shells using a chemical extraction process had a viscosity of 323 ± 15.60 mPa.s, indicating that biologically purified chitin gave chitosan with a high quality.

Keywords: *Bacillus thuringiensis* SA/ Chitin/ Chitosan/ Decalcification/ Deproteinization/ *Lactobacillus pentosus* L7/ Shrimp shells/ Viscosity

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LIST OF SYMBOLS

%	=	Percentage
σ	=	Shear rate
γ	=	Shear stress
η_{app}	=	Appearance viscosity
^{o}C	=	Degree Celsius

LIST OF ABBREVIATIONS

CFU	=	Colony forming unit
cm	=	Centimeter
cm^3	=	Cubic centimeter
DA	=	Degree of acetylation
DD	=	Degree of deacetylation
DP	=	Deproteinization
DC	=	Decalcification
EDTA	=	Ethylenediaminetetraacetic acid
g	=	Gram
GlcN	=	Glucosamine
GlcNAc	=	N-acetyl-D-glucosamine
Gyr B	=	Gyrase II
h	=	Hour
K	=	Kevin
L	=	Liter
M	=	Molar
mg	=	Milligram
ml	=	Milliliter
mM	=	Millimolar
min	=	Minute
nm	=	Nanometer
MRS	=	De Man Rogosa and Sharpe
NB	=	Nutrient broth
NA	=	Nutrient agar
Pa	=	Pascal
PCR	=	Polymerase chain reaction
PAGE	=	Polyacrylamide gel electrophoresis
PMSF	=	Phenylmethylsulfonyl fluoride
TTA	=	Total titratable acid
TSB	=	Tryptic Soy Broth
rpm	=	Revolutions per minute

s	=	Second
SDS	=	Sodium dedocyl sulfate
SHES	=	Shrimp head extract solution
WI	=	Whiteness index
µg	=	Microgram
µm	=	Micrometer

CHAPTER 1 INTRODUCTION

1.1 Background and Rationale

The main unutilized biomass from the shrimp packaging and processing industries is heads and body carapaces, which constitute 45 - 50% of the wet weight of fresh shrimps (Heu et al., 2003; Knorr, 1991; Omum, 1992; Sachindra et al., 2005; Teng et al., 2001). About 20 - 40% of shrimp bio-waste consists of chitin encrusted with calcium carbonate, protein, astaxanthin, and lipid residues (Venugopal, 2009; Xu et al., 2008). Shrimp bio-waste is often treated in landfills or discarded in sea water, resulting in ecological problems in coastal areas; whereas a small part is used as a major component in chicken or fish feed, mixed with other agricultural raw materials (Xu et al., 2008). The exoskeletons of crustacean waste from the sea-food industry are traditionally used to prepare commercial chitin and chitosan (Chang and Tsai, 1997; Mojarrad et al., 2007). Chitin, or β-(1, 4)-linked N- acetyl glucosamine (GlcNAc), is the most abundant natural polysaccharide on earth after cellulose (Felse and Panda, 1999; Khoushab and Yamabhai, 2010). Chitin can be converted to chitosan (β-(1, 4)- linked linear polymer of 2-acetamide-2-deoxy-β-D-glucose) by deacetylation with concentrated NaOH.

In commercial chitin extraction, chitin is usually isolated by a simple process which involves the use of alkaline and acid solutions to deproteinize and decalcify the shrimp shells. Chemical chitin extraction has a high efficiency for recovering purified chitin, but the process creates hazardous wastes which are harmful to human health and ecological systems (Kjartansson et al., 2006; Percot, 2003; Venugopal, 2009; Xu et al., 2008). In addition, the chemical process has a negative effect on the intrinsic and physical properties of purified chitin, leading to a decrease in the viscosity of chitosan (Xu et al., 2008). Crude proteins and carotenoids in the extracting solution are useless after deproteinization and decalcification (Kandra et al., 2012; Rao et al., 2000). Continued chitin production by chemical processes without development and utilization of novel technologies cannot solve the problem of achieving environmental sustainability (Kandra et al., 2012). Currently, strictness in environmental protection has become a basic requirement for waste management in food and agricultural industries.

To overcome the shortcomings of chemical chitin purification, several biotechnological techniques have been developed that are considered to be efficient alternative approaches for recovery of high quality chitin (Bautista et al., 2001; Jo et al., 2008; Kandra et al., 2012; Oh et al., 2007; Teng et al., 2001; Xu et al., 2008) . The use of commercial crude enzymes to extract chitin influenced the cost production and also gave the low extraction efficacy (Jo et al., 2008). As a substitute for chemical and enzymatic processes, lactic acid fermentation combined with microbial deproteinization warrant further investigation because they are eco-friendly and positive procedures. The objective of this work was to investigate a novel process for producing chitin from shrimp shells using biological process for decalcification and deproteinization, and produce chitosan from the created chitin.

1.2 Objective

The main objective of this research is to produce chitin and chitosan from shrimp shells using biological treatment processes.

The specific objectives:

1) To produce chitin from shrimp shells using biological processes
   (1.1) To isolate an effective lactic acid producing bacterium from Thai traditional fermented foods for using in the decalcification of shrimp shells
   (1.2) To isolate proteolytic bacterium producing proteases specific to shrimp protein for using in the deproteinization of shrimp shells
   (1.3) To create a two-step biological chitin purification: decalcification and deproteinization
2) To produce chitosan by deacetylation of chitin obtained from biological processes

1.3 Scopes of Work

1.3.1) Proteolytic bacteria and acid producing bacteria were isolated and used for chitin purification.

1.3.2) The efficacies of the selected bacteria to deproteinize and decalcify shrimp shells were investigated.

1.3.3) The cost effective bacterial culture media from seafood waste could be established.

1.3.4) The biological processes for producing chitin and chitosan from shrimp shells were testified.

1.4 Expected Results

1.4.1) A novel biological process to extract chitin from shrimp shells could be developed.

1.4.2) The biological purification process could preserve the intensive structure of chitin better than chemical process.

1.4.3) The good quality high viscous chitosan could be prepared.

CHAPTER 2 LITERATURE REVIEW

2.1 Shrimp Wastes

Shrimp wastes are inedible parts of shrimp consisting of heads and shells and can be obtained from shrimp industry, approximately 45 - 50% of the wet weight of fresh shrimps. The global production of crustacean wastes and chitin from crustacean processing industry is shown in Table 2.1. Shrimp wastes are often treated in landfills or discarded in sea water, resulting in ecological problems in coastal areas; whereas a small part is used as a major component in chicken or fish feed, mixed with other agricultural raw materials (Xu et al., 2008). Shrimp wastes contain three major components including chitin, protein, and carotenoids. The amount of each component in the shrimp waste has been previously reported as 17 – 35% chitin, 20 – 41 % proteins, 33 – 53% calcium on a wet weight basic (Mandeville et al., 1992; Shahidi, 1997; Synowiecki and Al-Klateeb, 2003). Amount of chitin in shrimp waste varied depending on types of waste (Table 2.2).

Shrimp wastes contain significant amount of carotenoids, particularly, red-orange colored pigment, astaxanthin. Shrimp accumulates carotenoids from the consumption of phytoplankton which is the major synthesizer of this pigment. Oxidation product of β-carotene (Astaxanthin) is major carotenoids in shrimp heads and shells. Astaxanthin, an oxidative product of β-carotene, has been reported as the major carotenoids in shrimp heads and shells. Astaxanthin can also attach with protein and lipid, called carotenoids complex. In addition, astaxanthin can be extracted and concentrated from shrimp wastes without losing of its functions and properties (Sachindra et al., 2005; Sachindra et al., 2007). Extraction process of astaxanthin from the shrimp waste can be done by either of following procedures, for example; pulverization, enzyme treatment, solvent extraction, and supercritical extraction using carbon dioxide. However, the disadvantages of these processes are high energy requirement to remove solvent, losing of pigment properties due to heating process, and present of residues solvent in the products. To overcome the drawbacks of those extraction processes, proteolytic enzymes were suggested as alternative approaches to hydrolyze and recover astaxanthin from the shrimp wastes (Shahidi, 1997). Since, astaxanthin is relatively insoluble in water, but it is soluble in

non-polar solvents and oils. Therefore, numerous solvents and natural oils were used to extract carotenoids from shrimp waste, for example in Table 2.3 shows astaxanthin extracted by soy oil and solvent from *Xiphopenaeus kroyeri* shrimp waste after enzyme treatment with proteolytic enzyme (Shahidi, 1997).

Table 2.1 Global availability of crustacean processing waste

Resource	Total landing	Waste available	Dry waste	Chitin content
Shrimp	1,292,476	516,900	129,475	32,311
Squid	398,219	99,531	24,882	1,244
Crabs	943,826	482,744	144,823	28,964
Krill	150,000	60,000	15,000	3,750

[Unit in metric ton (mt)]

Source: Venugopal (2009)

In shrimp heads, protein is found to be a minor component after lipid. Analysis of protein and carbohydrate in shrimp heads indicated that 17 amino acids consisting of Asp, Glu, Ser, Thr, Arg, Gly, Ala, Pro, Val, Met, Leu, Ile, Phe, Cys, Lys, His, Tyr, and proline; and 7 sugars consisting of ribose, xylose, fructose, mannose, glucose, glucosamine, and galactosamine were abundant and commonly found in shrimp heads. Now a day, shrimp heads are extensively used as a protein source for animal feed (Venugopal, 2009).

Table 2.2 Chitin content of selected crustacean and molluskan organisms

Species	Chitin content (%)
Shrimp head	11[a]
Shrimp shell	27[a]
Commercial shrimp waste	12-18[a]
Cancer crab	72.1[b]
Blue crab	14
Alaska shrimp	28
Clam shell	6.1
Oyster shell	3.6
Squid, skeleton pen	41

[a] ; wet body weight

[b] ; organic weight of the cuticle

Source: Venugopal (2009)

Table 2.3 Concentration of astaxanthin extracted by soy oil and solvent from shrimp waste

Enzyme	Sample	Astaxanthin (mg/100 g dry residue)	
		Oil	Solvent
No enzyme	Control	4.9	4.9
Alcalase	Insoluble fraction	5.15	11.39
Alcalase	Soluble fraction	1.71	-
Pancreatin	Insoluble fraction	4.71	10.8
Pancreatin	Soluble fraction	1.56	-

Source: Venugopal (2009)

2.2 Chitin

Chitin, poly (β-(1-4)-N-acetyl-D-glucosamine), is the second abundant natural polysaccharide on earth after cellulose, it was first identified in mushroom by Henri Braconot in 1811. The name is derived from the word “chiton” meaning a coat of nail. Chitin is synthesized by various numbers of living organisms such as bacteria, fungi, plants, and invertebrates. However, the main of commercial chitin source is the exoskeleton of marine organisms including lobster, crab, cuttlefish, shrimp, and prawn. The available chitin concentration in exoskeletons of marine organisms is higher than fungi, and plants. Therefore, chitin is usually isolated from sea food industry wastes. In nature, chitin is created for forming the outer protection coating (Exoskeleton) or function as a cell wall of yeasts and fungi, as crystalline microfibrils forming structural components. Chitin is slight difference from cellulose, hydroxyl at C2 position of glucose unit is replaced by acetamido group (Figure 2.1).

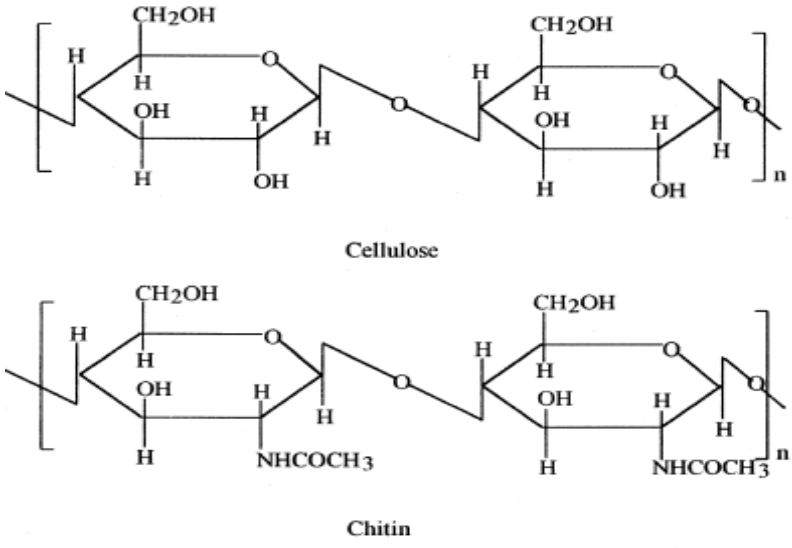


Figure 2.1 Structures of cellulose and chitin

In conventional process, chitin is extracted from crustacean shells by a simple process which involves the use of acid (HCl) and alkaline (NaOH) to eliminate calcium carbonate and protein from the shells. In addition, a decolorization step is often added to remove contaminated pigments in the product. This process must be adapted to each chitin sources; lobster, crab, cuttlefish, shrimp, and prawn due to ultra-structures of

initial materials have an effect on periods of interaction between chemicals and raw materials. The purified chitin needs to be graded in terms of purity and color depending on a residual protein and pigment which cause problems for utilization in further, especially for biomedical products.

2.2.1 Isolation of Chitin

The traditional chitin source is wastes from sea food industry; shrimp, crabs, squid, cuttlefish, krill, clams, and oyster. Raw materials for chitin production are contaminated with macro bio-molecules, and inorganic compounds. For industrial chitin isolation, chemical treatment processes need to eliminate contaminated compounds before chitin is used for various applications. Generally, the isolation process consists of three steps including decalcification, deproteinization, and blenching. In decalcification of the shells, shrimp shells are treated with diluted hydrochloric acid (HCl) for a period of 1 - 3 h. Concentrated acid may use for mineral removing such as 90% formic acid, 22% HCL, 6 N HCl, or 37% HCl (Venugopal, 2009). For the deproteinization step, the decalcified materials are treated with 4 - 5 M sodium or potassium hydroxides (NaOH or KOH) at 65 - 100^{o}C for 0.5 - 6 h and the ratio of decalcified materials to alkaline solution is about 1: 9 (10%, w/v). The treated period depends on kinds of materials, and amount of residual protein in the decalcified materials. After purification, chitin is washed and dried. The branching step is colorless process which can reduce amount of pigment in chitin. The chemical process for chitin extraction is shown in Figure 2.2.

The conditions for chitin extraction are determined by the required application of prepared chitin product. For example, removal of salts by a drastic decalcification can result in degree of acetylation in the product. Alkaline extraction for protein removal has an effect on chitin product by decease in degree of polymerization (DP) and degree of acetylation. Furthermore, crude protein in alkaline solution after deproteinization is useless due to the short of amino acid and undesirable reactions between amino acid occurring in a strongly alkaline medium, called racemization of amino acids. The extracted protein recovered in the form of hydrolyzate can be used as a flavoring agent and as a feed for fish or aquaculture (Venugopal, 2009).

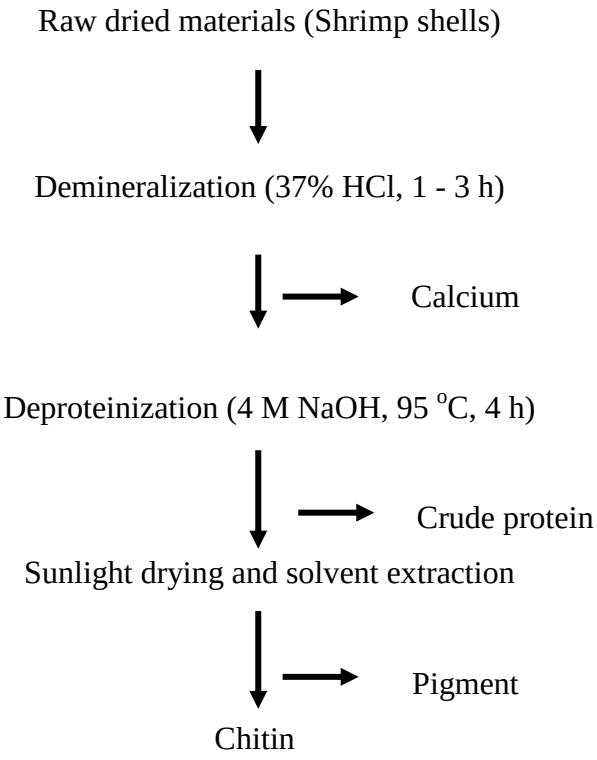


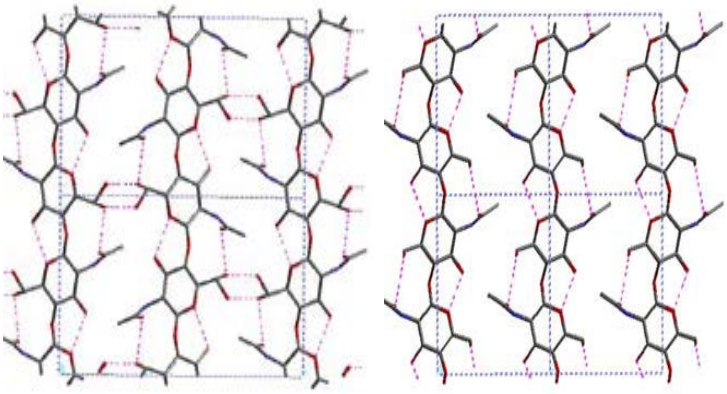
Figure 2.2 Process for chitin extraction

Chemical chitin extraction has a high effective to recover high grad of chitin. However, the process creates hazardous wastes which are harmful to humans and ecological systems (Kjartansson et al., 2006; Percot, 2003; Venugopal, 2009; Xu et al., 2008). Continued chitin production by chemical processes without development and utilization of novel technologies cannot solve the problem of achieving environmental sustainability. Therefore, the biological techniques for chitin purification have been investigated as desirable processes.

2.2.2 Properties of Chitin

Chitin has various physical properties such as very light, white or yellow wish powdery/flaky products. Like cellulose, chitin functions in the nature are a structural supporting polysaccharide, but chitin differs from cellulose in its properties, hydroxyl at position C2 of glucose unit is replaced with acetamido group. Chitin shows a strongly hydrophobic property. It is insoluble in water, several organic solvents, and in acidic or alkaline aqueous solutions. However, chitin can be slight soluble in hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in conjugation with aqueous solutions of mineral acids, and dimethylacetamide containing 5% (w/v) lithium chloride (Madhavan, 1992). The hydrolysis of chitin with concentrated acids or alkaline by breaking down glycosidic linkages releases N –acetyl -D-glucosamine, and bi or oligo - glucosamine units. The nitrogen content of chitin varies at 5 - 8% depending on the degree of acetylation. Chain lengths of chitin and degree of acetylation depend on isolation conditions and sources of raw materials. Chitin prepared from shrimp, crab, and lobster shells is generally used to be suitable for all proposes. Chemical derivatives of chitin have been produced from chemical reactions between various chemicals and native chitin. Properties of novel materials depend on chemical properties of substituted groups, e.g. carboxymethyl, hydroxyethyl, ethyl, chitin sulfate groups and others.

Natural chitins constitute two major allomorphs including α and β forms determined by infrared and solid-state NMR spectroscopy together with X-ray diffraction (Blackwell, 1973; Rudall and Kenchington, 1973). α - Chitin is various abundant in the nature; it can be found in cell walls of fungal and yeast, and in exoskeletons of various marine living organisms such as krill, lobster, crab, shrimp shells. The rarer β -chitin is found in association with proteins in squid pens (Rudall, 1969). A particularly β -chitin is found in the monocrystalline spines excreted from the diatom *Thalassiosira fluviatilis* (Dweltz et al., 1968; Herth et al., 1986). At the present, it has not been possible to obtain β -chitin from solution or by in vitro biosynthesis, whereas α -chitin can be synthesized by vitro biosynthesis, or enzymatic polymerization. The crystallographic parameters of α and β -chitin show that there is only one anti-parallel molecule per unit cell in α -chitin, while β -chitin has two parallel molecules per unit cell (Figure 2.3) (Gardner and Blackwell, 1975; Minke and Blackwell, 1978).



a b

Figure 2.3 Structures of α -chitin, (a) and β –chitin
Source: Gardner and Blackwell (1975); Minke and Blackwell (1978)

For β –chitin, it lacks hydrogen bonds between chitin sheets which β –chitin is found in the crystal structure. Therefore, sheets are tightly bound by a number of intra-sheet hydrogen bonds. This point indicates that various kinds of polar molecules such as water, alcohol, and amines, can easily diffuse in the crystal lattice of β -chitin without disturbing the sheet formation and the crystallinity of β -chitin. The inter-sheet swelling of α -chitin crystals is very specific for binding between chitin sheets which water and alcohols cannot penetrate the crystalline lattice of α -chitin. Unlike β -chitin, the guest molecules are incorporated between the chitin sheets of α -chitin. This point indicates that α –chitin cannot be soluble in water. However, α -chitin can be swollen in the concentrated acid due to hydrogen bonds between chitin sheets are destroyed.

2.2.3 Chitin Derivatives and Applications

Like cellulose, properties of chitin derivatives depend on substituted groups replaced hydroxyl at C2, C3, and C6. The most important derivative of chitin is chitosan (Figure 2.4). Chitosan are commercially produced by partial deacetylation of chitin polymer with concentrated NaOH or enzymatic hydrolysis (Chitin deacetylase).

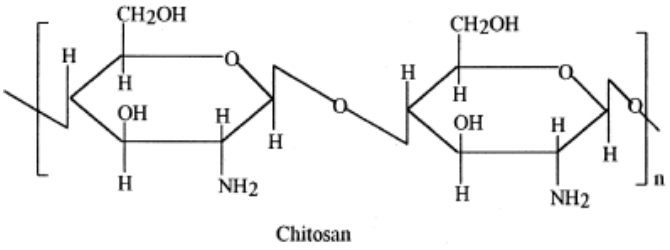


Figure 2.4 Structure of chitosan

After chitosan, the most studied derivative of chitin is carboxymethylchitin (CM-chitin is anionic polymer). Unlike the native chitin, CM-chitin can be soluble in water. CM-chitin can be produced from same reaction used for preparation of CM-cellulose. Briefly, chitin is treated with monochloracetic acid in the presence of concentrated sodium hydroxide. The same method can be used for carboxymethylation of chitosan. Other derivatives such as fluorinated chitin (Chow and Khor, 2001), Nand O-sulfated chitin (Tokura et al., 1994), (Diethylamino) ethylchitin (Kurita et al., 1998), phosphoryl chitin (Andrew et al., 1998) and chitin carbamates (Vincendon, 1992) have been prepared in the previous studies. Almost chitin derivatives are more soluble in water than native chitin. Chitin is hydrolyzed with concentrated acid or chitinase under optimal conditions to obtain series of oligo-chitins, chitin oligomers (Rupley, 1964). Chitin oligomers have many advantages such as anti-tumor, bactericidal, and fungicidal activity. Chitin oligomers are also used as substrate for testing lysozyme activity. They are also used as active starting blocks to be grafted on protein and lipids to obtain analogs of glycoproteins and glycolipids (Rinaudo, 2006).

Chitin and derivatives have diverse applications in agriculture, biotechnology, chemistry, cosmetic, dentistry, food product development, medicine, textile, and veterinary sciences (Venugopal, 2009). Chitin shows low toxicity and inert substance leading to a good material for producing pharmaceutical products. The applications include nonwoven artificial skin for burn, biodegradable suture, and as the vibrating panel in audio speakers. Chitin has been used to prepare chromatography column to separate and detect lectins (Datta et al., 1984). 6-O-carboxymethyl chitin activates function of macrophages, and suppresses tumor cells or cancer cells in mice. Chitin is widely used to create immobilize enzymes for various food industries such as clarification of fruit juices (Krajewska, 2004). Immobilize enzymes with chitin can

extend half-life and be easy for recovering enzymes. Chitin has many applications in other areas such as in waste water treatment, absorption of silver thiosulfate complexes, and actinides (Kosyakov et al., 2002; Songkroah et al., 2004). Chitin can be produced in the formation of films, and fibers. Chitin derivative fibers are used for the paper making process to improve breaking strength of paper (Krajewska, 2004). However, the main purpose of chitin film and fiber application is use for medical and pharmaceutical applications, such as wound-dressing material and controlled drug release (Krajewska, 2004). In agriculture, chitin contributes to retention of nutrient in the soil (Venugopal, 2009). Chitin is hydrolyzed by decomposers to produce ammonia which takes part in the nitrogen cycle. Some time, chitin was used to activate host plant resistance against plant pathogens (Venugopal, 2009).

Table 2.4 Application of chitin biomaterials

Form of chitin	Species	Application clinical case
Cotton	Dog	Abscess, Bite wound, Contused wound, Fracture
	Cat	Abscess, Bite wound, Contused wound
	Human	Decubotus ulcer
Sponge	Cow	Decubotus ulcer
		Abscess, Arthritis, Contused wound
	Dog	Abscess, Bite wound, Contused wound
	Cat	Abscess, Bite wound
	Rabbit	Abscess
	Monkey	Bite wound
	Human	Skin and soft-tissue defect
Composite with NFF	Cow	Fetlock deformity, Capsuloplasty, Tendoplasty, Redressement of entopion
	Cat	Skin defect
Powder	Dog	Contused wound
	Cat	Bite wound
	Human	Surgical wound, Slow healing Surgical incision, Perineal wound, Excision of a keratosis, Ulcer

2.3 Chitosan

Chitosan is a name of de - N - acetylated chitin chain, poly-(β-(1-4)-linked-2-amino-2-deoxy-D-glucose. A number of degrees of deacetylation in chitosan are various differences depending on prepared conditions. Generally, chitosan requires at least 70% of degree of deacetylation (DD). Chitosan was discovered by Rouget in 1859. However, interests in chitosan applications slowly begin in the 1930s and early 1940s. Commercial chitosan is produced by deacetylation of chitin in 30 – 60% sodium or potassium hydroxide solutions at 80 - 140^{o}C. Figure 2.5 represents process for chitosan preparation. Characteristics of chitosan in terms of molecular weight, and degree of deacetylation depend on treatment conditions used for deacetylation. High temperature can enhance deacetylation efficacy. However, chitosan polymers may be fragmented during preparation under hard condition.

Chitosan is a pseudo-natural cationic polymer which is soluble in aqueous acidic media due to protonation of the $–NH_2$ function on the C-2 position of the D-glucosamine repeat units. Swollen chitosan is prepared by dissolving chitosan powder or flakes in the diluted acetic acid and precipitated by addition of alkaline solution to deprotonate and create the insolubility of the polymer at neutral pH.

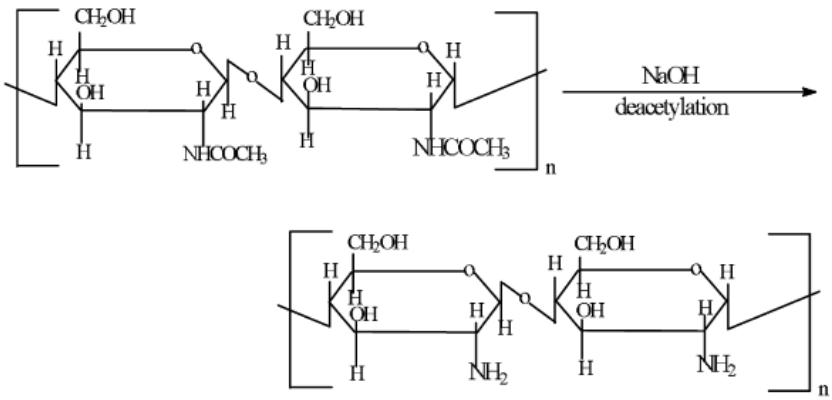


Figure 2.5 The chemical process for chitosan production

The soluble properties of a chitosan are related with degree of deacetylation (DD), and molecular weights. In addition, solubility of chitosan influences ionic concentration and salting-out effect of solution. The solubility of chitosan is usually tested in acetic acid by dissolving in 1% or 0.1 M acetic acid. The concentration of needed protons is at least equal to the concentration of -NH2 units. Therefore, the amount of acid required to dissolve the chitosan can be used to determine degree of deacetylation (Rinaudo et al., 1999).

2.3.1 Properties of Chitosan

Chitosan is a cationic polyelectrolyte, white solid. Chitosan cannot be soluble in pure water. However, Chitosan is soluble in dilute acidic solutions. A minimum deacetlylation of chitosan requires at least 70% (DD), is acceptable for numerous applications. The molecular weight of natural chitosan is higher than 1 mDa, whereas commercial chitosan is about 0.1 - 0.2 mDa (Shahidi and Abuzaytoon, 2005). Decolorization treatment of chitosan has an effect on viscosity property due to change in molecular weight of chitosan. Therefore, decolorization is not essential for acceptation of chitosan in term of quality parameter. Chitosan derivatives in form of acetate, ascorbate, lactate, and malate are soluble in water. The pK_a value of the positive charged ammonium group is about 6.2. If pH value of acidic solution increases to 6.5, chitosan is precipitated in the form of gel. Chitosan has a very potential to form a complex structure with several cationic metals. There are many factors which influent efficiency of complex formation such as pH and metal content in solution, physical state of chitosan (powder, gel, fiber, and film), degrees of deacetylation in chitosan chain, amount of $–NH_2$ distribution and DP of oligo – chitosans. The complex usually starts to form when DP > 6 (Rhazi et al., 2002). In the natural, free electron in cation is very important for the mechanism of interaction (Krajewska, 2004). The affinity of chitosan for cations absorbed on film shows selectivity following the order, $Cu^{+2} >> Hg^{+2} > Zn^{+2} > Cd^{+2} > Ni^{+2} > Co^{+2} \sim Ca^{+2}$ and $Eur^{+3} > Nd^{+3} > Cr^{+3} \sim Pr^{+3}$, for divalent and trivalent cations. Some cases, chitosan powder was dispersed in silver nitrate solution or used to fill a column to adsorb mercuric ions from a chloride solution (Peniche-Covas, 1992). However, chitosan is a very useful for copper absorption in liquid waste water treatment.

Chitosan can be hydrolyzed with specific enzyme, called chitosanase. However, non – specific enzyme such as lipase and amylase may degrade chitosan. Chitosan gives a highly viscous solution when it is solubilized in 1% acetic acid. The viscosity of chitosan relates with molecular weight and degree of polymerization. The viscosity of 1% chitosan in 1% acetic acid is often used to determine the quality of chitosan. Higher viscosity indicates that high quality of chitosan is obtained. Chitosan in microcrystalline form is characterized by various advantages compared with standard chitosan. These include high stability, water retention, biodegradability, high film forming capacity and high chelating property. Chitosan derivatives can be prepared by Millard – type reaction which improve solubility at pH ≥ 7. The modified chitosan shows a greater solubility than native chitosan. For example, chitosan – maltose derivative dissolve in water at pH 10. Table 2.5 represents the specifications for chitin and chitosan.

Table 2.5 Specifications for chitin and chitosan

Properties	**Chitin food grade**	**Chitosan pharmaceutical grade**	**Liquid chitosan technical grade**
Appearance	White/yellow Flask	White/yellow Powder/flaks	Clear yellow Liquid
Odor and taste	Odorless, tasteless	Odorless, tasteless	Odorless, tasteless
Moisture (%)	< 10	< 10	< 10
Ash (%)	< 2.5	< 0.2	< 10
Protein (%)	< 1.0	< 0.3	< 10
Deacetylation (%)	-	70-100	> 90
Viscosity (0.5% solution; cP)	600	< 5	50
pH	7.9	7.9	<5.5
Heavy metal (arsenic and lead; ppm)	< 10	< 10	< 10

2.3.2 Chitosan Derivatives

Main property of chitosan derivatives is better soluble in water than native chitosan, whereas native chitosan is insoluble in water. This property indicates the function of a new material for future application. Chitosan derivatives are often used for various purposes. Carboxymethyl chitosan (CM-chitosan) is common chitosan derivatives. Like cellulose and chitin, CM-chitosan can be produced by treating chitosan in mono-chloroacetic acid solution and concentrated sodium hydroxide. CM-chitosan is water soluble in wide ranges of pH. N-methylene phosphonic chitosans is a good anionic derivative which can form a complex with many cations such as Ca^{2+}, Cu (II), Cd (II), Zn (II) etc. (Varum, 1991). The complexion protects corrosion on surfaces materials. This derivative is modified and added with alkyl chains to create amphiphilic property used in cosmetics as emulsifier. Trimethyl -chitosan ammonium produced by treating chitosan in methyl iodide and sodium hydroxide under controlled conditions is a water soluble cationic derivative. These polymers show good flocculating properties with kaolin dispersions suggested in many applications to paper making (Domard et al., 1989).

Carbohydrates can connect with chitosan backbone at the C-2 position by reductive alkylation, called carbohydrate branched chitosan. These derivatives are important in medical purposes due to they have specific recognized with lectins and could be used for drug targeting (Morimoto et al., 2002). Alkylated chitosans, these derivatives are very important as amphiphilic polymers. The first derivative is C-10-alkyl glycoside branched chitosan. However, amphiphilic properties of these derivatives depend on chain lengths of substituted group (C_n from 3 to 14) and controlled DS (Not least than 10% of DS to maintain water solubility in acidic conditions). Alkylated chitosans is high solubility in acid solution (pH< 6). The main property of alkylated chitosans is surfactant. Alkylated chitosans have an effect on the surface tension and improve the stability of the interfacial film. And other properties, alkylated chitosans increase the viscosity of aqueous solution due to chain length and amount of DS.

2.3.3 Applications of Chitosan

Table 2.6 represents applications of chitosan and derivatives. The application potential of chitosan is multidimensional such as in food and nutrition, biotechnology, material science, drugs and pharmaceutical, agriculture and environmental protection, and recently in gene therapy.

Table 2.6 Principal applications for chitosan

Types of application	Functions
Agriculture	Defensive mechanism in plants, Stimulation of plant growth, Seed coating, Frost protection, Time release of fertilizers and nutrients into the soil
Water & waste treatment	Flocculant to clarify water (drinking water, pools), Removal of metal ions, Ecological polymer (eliminate synthetic polymers), Reduce odors
Food & beverages	Not digestible by human (dietary fiber), Bind lipids (reduce cholesterol), Preservative, Thickener and stabilizer for sauces, Protective, fungistatic, antibacterial coating for fruit
Cosmetics & toiletries	Maintain skin moisture, Treat acne, Improve suppleness of hair, Reduce static electricity in hair, Tone skin, Oral care (toothpaste, chewing gum)
Bio pharmaceutics	Immunologic; antitumoral, Hemostatic and anticoagulant Healing; bacteriostatic

Food

Chitosan is usually used as a food additive in food technology. There are various roles in food production such as antimicrobial, and antioxidant. Chitosan film can coat on the surface of food. Obtained film decreases oxygen permeable and suppresses microbial growth. The mechanism of antioxidant action derives from metal complexion property and also its ability to form a complex with lipids in food. Roles of chitosan in food are focused on improvement of quality and shelf life of various foods from agriculture, poultry and sea food origins (No et al., 2007).

Degree of polymerization in chitosan has an effect on rheological properties by increasing viscosity in foods. Other applications of chitosan gels in food technology include clarification of wine and vinegars, and in waste water treatment. Chitosan and chitin are used as food additives in cookies, bread, and noodles for improvement of food texture. Chitosan can inhibit the growth of several microorganisms in food such as *Aeromonas hydrophila*, *Bacillus cereus*, *B. licheniformis*, *B. subtillis*, *Clostidium perfringens*, *Brochothrix spp*., *Lactobacillus spp*., *Listeria monocytogenase*, *Pseudomonass spp*., *Salmonella typhimurium*, *S. enteridis*, *Serratia liquifaciens*, and other including yeast (*Candida* spp. and *Saccharomyces* spp.) and mold (*Aspergillus* spp., *Penicillium* spp., and *Rhizopus* spp.). The antimicrobial activity of chitosan depends on types and concentration of chitosan. For Gram negative bacteria, chitosan disrupts the barrier properties of outer membrane (Knorr, 1984; No et al., 2007; Sagoo et al., 2002).

Force of ionic interaction between surface of Gram negative bacteria (Negative charge) and positive charge of chitosan cause deterioration of the cell membrane. Microorganisms cannot uptake nutrients to cells and maintain cell functions leading to inhibition of microorganism growth (Helander et al., 2001). In addition, cupric ions-chitosan complex can interfere with microbial growth and toxic for many microorganisms. For fungi, chitosan coated on surface of food and plants inhibits gas permeable. It has an effect on fungal growth, stimulation of many defense processes including accumulation of chitinase, and production of proteinase inhibitors. Carboxymethyl chitosan inhibited iron –activated auto-oxidation by chelating action. Chitosan may be uses to extend the shelf life of food under chill storage of cod, pork

sausage, and fish patties (Venugopal, 2009). The antimicrobial properties of chitosan have various advantages. Chitosan is used together with other food preservative techniques such as chilling, modified atmosphere packaging, or high pressure. Like chitosan, chitosan oligosaccharide shows the antimicrobial activity. About 5% of chitosan oligosaccharide inhibited growths of pure pathogenic and spoilage bacteria in synthesis medium such as *Bacillus cereus, Lactobacillus brevis, Leuconostroc mesenteroides, Micrococcus varians, Staphylococcus aureus, Acinetobacter spp., E.coli, Pseudomonas aeruginosa, Salmonella typhimurium,* and *Serratia liquefacience.* High molecular weight of chitosan oligosaccharide can inhibit the growth of fungal and yeasts such as *Candida albicans, C. versatiles, Rodotorula glutinis, S.cerevisiae, Trichosporom pullulans,* and *Aspergillus niger*.

Food contains significant amount of unsaturated fatty acid leading to development of lipid oxidation. Rancidity in food leads to loss of freshness and finish the shelf life of food. Chitosan and its derivatives can reduce and inhibit lipid oxidation. Antioxidation properties depend on amount and kinds of chitosan, storage temperature, and molecular weights of chitosan. For example, N- carboxymethyl chitosan combined with lactate, acetate, and pyrolidine carboxylate salts could extend a shelf life period of cooked meat when stored at refrigerated temperature. The result revealed that cooked meat kept freshness until nine day after storage under selected condition. Table 2.7 represents application of chitosan in food product.

Water treatment

Chitosan and its derivatives in forms of carboxymethyl chitosan and cross-linked chitosan are often used in waste water treatment to remove heavy metal ions. For drinking water, chitosan and its derivatives are used for lead, copper and cadmium removal. The mechanism of metals removal by chitosan is complex formation between amino groups and heavy metal ions. Chitosan and its derivatives have higher an efficient than activated charcoal for removing polychlorinated biphenyls from contaminated water. The hydroxamic acid derivatives of chitin and chitosan are most efficient in removing lead and copper (Hirotsu et al., 1986)

Biotechnology

Chitosan is used as a matrix for immobilization of enzymes and microbial cells. Various published papers demonstrated using immobilized enzyme for multiplicity of applications ranging from wine, sugar, and fish industry to organic compounds removal from wastewater to sophisticated biosensors for both in situ measurements of environmental pollutants, and metabolic control in artificial organs. In pharmaceutical applications, chitosan is used for drug and gene deliveries. Cationic derivatives are recognized to enhance the adsorption of drugs at neutral pH. *N*-Lauryl-carboxymethyl chitosan is an amphiphilic polymer. It forms micelles that solubilize taxol, making it more effective therapeutically, and it is found to be safe in terms of membrane toxicity. This type of derivative is generally useful as a carrier for hydrophobic cancer drugs. Chitosan and its derivatives are used for gene transfection. *N*-alkylated chitosan showed that transfection efficiency increased upon elongating the alkyl side chains and levels off when the number of carbons in the side chain exceeds 8.

Table 2.7 Applications of chitosan in food products

Products	Benefits
Bread	Chitosan coating controls moisture loss and lipid oxidation, helps control starch retro-gradation by inhibiting microbial growth.
Egg	Chitosan films protect moisture and CO_2 loss and internal quality, shelf life increase.
Fruit and vegetables	Control respiration, Antifungal activity helps control of decay.
Fruit juices	Clarification of apple, lemon, grape and orange juices. Control acidity and inhibits yeasts
Mayonnaise	Enhances-oil-in-water emulsion and stability
Meat	Antioxidant and antimicrobial activities extend shelf life of chilled meat, patties, sausages and intermediate moisture product. Irradiated (2.5 kGy) chitosan has superior antioxidant activity. It can reduce nitrate in sausages.
Seafood products	Antioxidant and antimicrobial properties extend chilled shelf life.
Dairy products	Antimicrobial activity extends chilled shelf life of milk.

2.4 Carotenoids

Carotenoids are a family of fat soluble compounds. They provide red or orange colors to plant, algae, and cyanobacteria. Several vegetables have large amounts of carotenoids. The functions of carotenoids in plant, algae, and cyanobacteria are light harvesting and protecting in these photosynthetic tissues. Carotenoids are synthesized by photosynthetic organisms including plant, and also some bacteria and fungi. Animal including human, chicken and other animals cannot synthesize carotenoids. Over 600 carotenoids have been discovered and identified (Venugopal, 2009).

2.4.1 Properties of Carotenoids

The carotenoids can be separated into two categories including hydrocarbon compounds (β carotene and xanthophylls) and the oxygenated derivative of carotenoids (astacene, astaxanthin, canthaxanthin, cryptoxanthin, echionine, lutein, neoxanthin, violaxanthin and zeaxanthin). β carotene ($C_{40}H_{56}$, molecular weight of 536.9) which is one of abundant carotenoids in nature is a polyunsaturated hydrocarbon and primary source for producing provitamin A. Astaxanthin is 3,3’ dihydroxy-β, β'- carotein-4,4' dione and canthaxathin, β,β' - carotein-4,4' dione. The isomers differ not only in their melting points, solubility, and stability, but also in respect to absorption affinity, and color intensity. Carotenoids cannot be soluble in water. They are soluble in most organic solvents and oils. They are sensitive to oxidation, isomerization, and polymerization when dissolving in dilute solution under light and in the presence of oxygen. They can store in cool place, in a tight, light resistant container containing inert gas without changing significant of properties and functions. Figure 2.6 shows chemical structures of some important carotenoids.

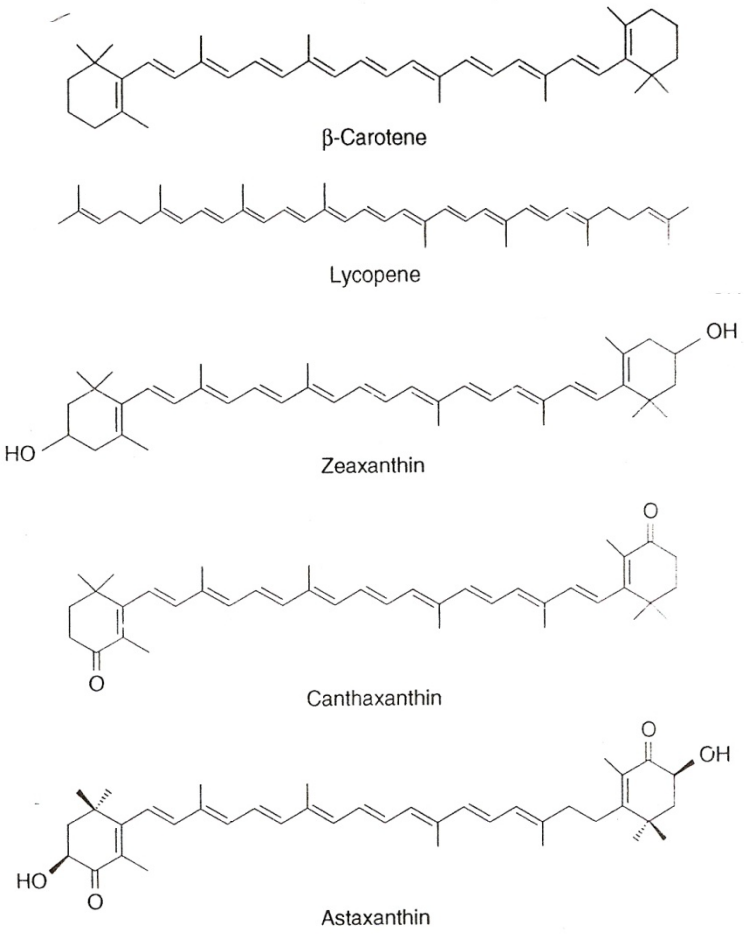


Figure 2.6 Chemical structures of some important carotenoids

2.4.2 Isolation of Carotenoids

Marine sources of carotenoids can be divided into two major categories including algae, and marine fishery sources. Most algae contain chlorophylls, carotenoids and phycobiliprotein. Carotenoids help marine macro-algae survive in shallow coastal water by offering efficient photosynthesis. Red algae don't contain universal carotenoid compositions. Aquatic animals consist of significant amounts of carotenoids, the red-orange pigments (Astaxanthin). The primary source of carotenoids for fish and shellfish is algae. Secondary sources are marine animals accumulated the pigment from their consumption of phytoplankton and subsequently consumed by other marine animals.

The most commercially harvested crustaceans such as crab, shrimp, and prawn contain significant amounts of carotenoids in their head and shell portions. Concentration and types of pigments found in crustaceans depends on kinds of source. Table 2.8 represents carotenoid content and composition in various wild species of penaeidae.

Table 2.8 Carotenoid content and composition in various wild species of penaeidae

Carotenoids	***P. vannamei***	***P. monodon***	***P. japonicus***	***Metapenaeus monoceros***
Composition	%	%	%	%
β- carotein	0.5	0.2	0	3.0
Yellow xanthophylls	30.0	0.4	19.0	23.0
7,8Didehydroastaxanthin	0.5	1.0	2.0	2.0
Astaxanthin total	65.5	98.0	79.0	72.0
Diesters	50.8	27.5	46.8	30.5
Monoesters	46.6	58.2	36.7	52.8
Free form	4.6	14.3	16.5	16.7

Carotenoids can be extracted and concentrated from shrimp processing waste without losing of functions and properties (Sachindra et al., 2005; Sachindra et al., 2007). In commercial processing, dried shrimp processing wastes are subjected to processes that include pulverization, enzyme treatment, solvent extraction, and supercritical extraction using carbon dioxide (Sachindra et al., 2005; Sachindra et al., 2007). However, these processes have several disadvantages such as high energy requirement to remove solvent, losing of properties of pigment by heating process and present of residues solvent in the product. The biological processes have been suggested to recover carotenoids from shrimp waste. For example, using proteolytic enzymes such as Trypsin in the presence of ethylenediamine tetra acetic acid (EDTA) at pH of 7.7 and 4^{o}C can hydrolyze and recover carotenoid up to 49% from carotenoid proteins (Venugopal, 2009). In addition, other enzymes such as Alcalase and Pancreatin have also been used for this purpose. The results showed that Alcalase was more efficient than Pancreatin. Alcalase could increase the recovery of astaxanthin from 4.7 to 5.7 mg/100g of dry waste and degree of hydrolysis of waste when 12% Alcalase was added (Chakrabarti, 2002; De Holanda and Netto, 2006). However, trypsin can more recover astaxanthine from shrimp heads than Alcalase and Pancreatin. During extraction process, carotenoids may require antioxidants. These compounds not only protected the pigments against oxidation, but also helped them to maintain the bright red orange color of carotenoid protein complex (Chakrabarti, 2002).

2.4.3 Applications of Carotenoids

The major advantage of carotenoids is antioxidant activity and precursors of vitamin A. These functions provide protection to living organisms against various diseases. Dietary carotenoids are considered as beneficial compounds in the prevention of diseases such as cancers and eye disorders. They are precursors of vitamin A and have a role in protecting the immune system and cellular health in the body.

Functions of carotenoids in aquaculture

In the present, carotenoids were used as components of feed in aquaculture industries. They have various functions in aquaculture feed such as pigmentation, antioxidant functions, as dietary source of provitamin A, cellular protection from photodynamic damage, enhancement of growth and reproductive potentials. Astaxanthin is widely used in salmonid and crustacean aquaculture to provide the pink color characteristic of that species.

Poultry feed

Canthanxanthin alone or together with astaxanthin are also used as a source of pigment for broilers to improve the color of egg yolk and also to increase a yellow hue of skin. The color of egg and skin of broilers is related with concentration of pigment in animal feed. However, this relation is not linear. Many nutritional, physiological and environmental factors involved in the process of canthanxanthin deposition that explains the great variability of the results obtained in practice, especially for fish. The Food and Agriculture Organization of the United Nation/World Health Organization (FAO/WHO) joint Expert Committee on Food Additive (JECFA) (1966) assessed intake canthanxanthin supplied through animal feed and found an acceptable daily intake (ADI) of 0.03 mg/kg body weight. Canthanxanthin has very low toxicity. The algal xanthophylls, peridinin, fucoxanthin and other carotenoids have also used as yolk-pigmenting agent for the poultry (Anderson et al., 2006).

2.5 Proteases

2.5.1 Classification of Protease

Proteases are commercially available enzymes produced approximately 40% of the total worldwide production of enzymes in every year (Haddar et al., 2009). Proteases are hydrolytic enzymes which show degradation of protein or polypeptide chain. Proteases can break down peptide bonds to release free amino acids, or decrease a size of polypeptide chain. Proteases can be produced from bacteria, fungi, and animals. However, bacterial proteases are a critical important source for several industries. Proteases have various applications often used in various industries such as in laundry detergent, food, pharmaceutical, leather, silk, and for recovery of silver from used X-ray films (Gupta et al., 2002; Haddar et al., 2009; Kumar and Takagi, 1999). Proteases can be classified into 3, or 4 subcategories depending on basic of their pH sensitive (Acid protease, Neutral protease and Alkaline protease) and on the nature of catalytic site namely, metallo- (EC.3.4.24), aspartic- (EC.3.4.23), cysteine- or sulphydryl- (EC.3.4.22), or serine-type (EC.3.4.21). Classification of protease and microbial occurrences are shown in the Table 2.9. However, alkaline proteases including serine protease and metallo-protease are particularly important because they are both stable and active at high pH solutions and in the presence of surfactants and oxidizing agents (Rao et al., 1998).

Like other enzymes, there are 2 major actions of protease on protein structure including endoprotease (Endopeptidases) and exoprotease (Exopeptidases). Endopeptidases cleave polypeptide chain randomly at internal sites which they degrade polypeptide chain to low molecular mass multi-mers of polypeptide, whereas exoproteases hydrolyze polypeptide chain and create one amino acid from N-terminus (Amino peptidases) or from C terminus (Carboxypeptidases). Exopeptidases, especially aminopeptidases, are ubiquitous, but less readily available as commercial products, since many of them are intracellular or membrane bound (Klomklao, 2008). Figure 2.7 shows actions of endopeptidases and exopeptidases on protein structure.

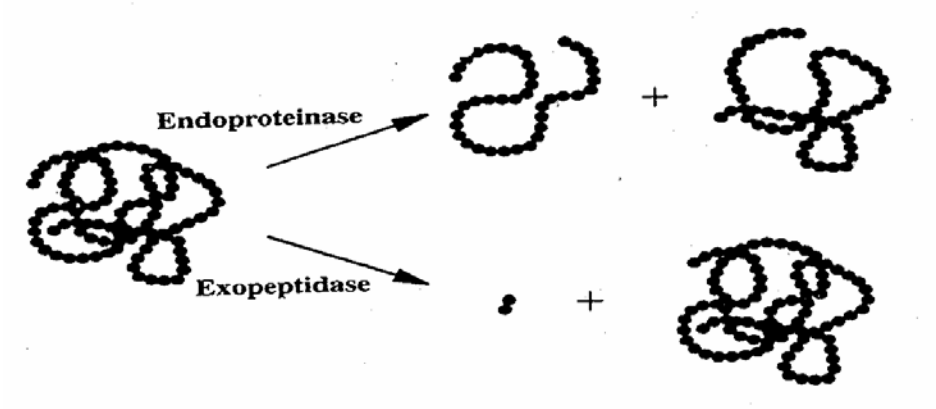


Figure 2.7 Actions of endopeptidases and exopeptidases on protein structure

Alkaline proteases are defined as proteases which are active in a neutral to alkaline pH ranges. Serine proteases and metalloprotease are found that they have strongly activity under alkaline and neutral conditions. The alkaline serine proteases are the most important group of enzymes exploited commercially. Serine proteases have been described as a group of endoproteases and exoprotease containing nucleophilic serine residue located in their active site. This protease differs from other proteases by the present of aspartate and histidine residues which, along with the serine, forms the catalytic triad. They can operate in neutral and alkaline pH, with optima at pH 7–11. They show broad substrate specificity. They showed generally in low molecular mass (18–35 kDa). For example, serine proteases are often used in biotechnologies or industries namely chymotrypsin-like proteases, subtilisin-like proteases or subtilases and trypsins. The metalloproteases are hydrolytic enzymes which activity depends on the presence of bound divalent cations. Chemical modification studies suggest that there may be at least one tyrosyl residue and one imidazole residue associated with the catalytic sites of metalloproteases (Whitaker, 1994). The metalloproteases are inhibited completely by chelating agents such as 1, 10-phenanthroline, EDTA, and sometimes by the simple process of dialysis. Molecular weight of metalloproteases are generally about 37-41 kDa for extracellular metalloprotease, and large molecular weight of metalloprotease are usually detected in intracellular.

Table 2.9 Classification of protease

Protease category	Type	Sub-types	Microbial occurrence	Conditions for optimal activity
Exopeptidases (site of action) Aminopeptidase	Majority intracellular,	-	Bacteria and fungi	-
Carboxypeptidases	Extracellular and intracellular	Serine carboxypeptidases, metallocarboxy peptidases,and cysteine carboxypeptidases	Bacteria and fungi	Variable
Endopeptidases (catalytic mechanism) Serine proteases	Extracellular and intracellular	Serine alkaline type—largest subgroup	Viruses, bacteria, and eukaryotes	Neutral and alkaline pH, with an optimum between pH 7 and 11, broad substrate specificities
Aspartic proteases	Extracellular and intracellular	Pepsin and rennin-like enzymes, acidic enzymes	Viruses, bacteria, and fungi	pH 3–4 and isoelectric points in the range of pH 3–4.5, molecular masses 30–45 kDa.
Cysteine/thiol proteases	Extracellular and intracellular	Papain-like; trypsin-like with preference for cleavage at the arginine residue; specific to glutamic acid; and others	Prokaryotes and eukaryotes	Normally, neutral pH But also, pH 4.9–8.4
Metalloprotease	Extracellular and intracellular	Neutral; alkaline; Myxobacter I; and Myxobacter II	Prokaryotes and eukaryotes	pH 4.0–10.0

Source: Brar et al. (2007)

2.5.2 Protease Production

Isolation of proteolytic microorganisms

To develop an industrial enzyme, the isolation and screening process to obtain microbial strain are very important stage to produce the enzyme in commercial yields. Extracellular microbial products, like enzymes, are screened using a plate assay and the organism's production can be related to the zone of hydrolysis (of the substrate) around the colony. A plate assay for the screening proteolytic microorganisms using various different agar media containing protein substrates such as gelatin, bovine serum albumin, and hemoglobin are normally used. However, a plate assay has a weak point due to enzymes such as proteases may be been inducible or repressible by synthesis media. After isolation of suitable strain, it is necessary to increase enzyme production by optimization of process parameters, such as production media – compositions, pHs, volumes, moisture contents, concentration of mineral salts, age and size of the inoculum, fermentation time, temperatures, carbons, organics, and inorganic nitrogen supplements (Sumantha et al., 2006).

Media composition

To produce proteolytic enzyme, media containing a rich nitrogen sources are important to get high protease yield. Soybean meal, casein, gelatin, corn steep liquor, distiller's soluble and brewer's yeast were used to produce protease (Sumantha et al., 2006). High carbohydrate concentrations in protease production media could repress enzyme production and the present of free amino acids also have a negative effect on protease production (Ivanita et al., 1978). Isoleucine and proline repress protease production by *Bacillus* sp., while polypeptides and native proteins have the positive effect on protease production.

Fermentation

The nature of the fermentation, solid or submerged, has an effect on growth of proteolytic microorganism and enzyme production (Sumantha et al., 2006). Depending on the culture conditions used, two different forms of the same enzyme may be also expressed, such as the two acid proteases produced by the solid-state fermentation (SSF) of bran by *Aspergillus oryzae*, which differed in their carbohydrate content, one being a glycoprotein and the other devoid of any carbohydrate (Tsujita and Endo, 1976).

Submerged fermentation (SmF)

Submerged fermentations (SmF) are process which substrate is dissolved or remains suspended in an aqueous medium. SmFs carried out with different substrates result in varying protease activities. In generally, simple nitrogen sources such as casein and gelatin result in low units of enzyme, whereas complex substrates such as soybean meal and wheat bran result in higher protease activities (Sumantha et al., 2006). Supplementation of a nitrogen-rich medium with carbon source, glucose also activates protease production. There are many types of SmFs including batch, fed-batch, and continuous. Continuous process was found that it could produce a high yield. However, an enzyme unit was lower than use of the batch fermentations (Aleksieva and Mutafov, 1997).

Solid-state fermentation (SSF)

Solid-state fermentations (SSF) involve microbial modification of a solid, undissolved substrate, in which microbial cultures are grown on a moist solid with little or no free water, although capillary water may be present (Sumantha et al., 2006). Concentration of solids is generally high, whereas water content is usually low. With regard to cost economics, SSF has been proved to be more efficient than SmF (Pandey, 2003). The product can be recovered in highly concentrated form as compared to those obtained by submerged fermentations.

Immobilization

Immobilization matrices including the conventional supports such as alginate, agar, carrageenan, polyacrylamide, glass beads, polyurethane foam, metal surfaces, *etc* are used to prepare immobilize cells. Cheap supports such as vermiculite have been used to immobilize neutral protease by adsorption. Immobilized enzymes have been found to retain their activity better than their free enzyme. In addition to enzymes production, immobilized microbial cells of bacteria or the mycelia of fungus are found to produce higher yields of enzyme than the non-immobilized ones (Aleksieva et al., 1988; Zamost et al., 1990)

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2.5.3 Applications of Proteases

Alkaline proteases account for a major share of the enzyme market all over the world. Alkaline proteases from bacteria find numerous applications in various industrial sectors and different companies worldwide have successfully launched several products based on alkaline proteases (Table 2.10).

Food and feed industry

Traditionally, microbial proteases have often been used in the food industries in many fields. Protein hydrolyzate was usually prepared by alkaline proteases to obtain the product with high nutritional value. The protein hydrolyzates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks (Neklyudov et al., 2000). The basic function of proteases is to hydrolyze proteins; and this property has been used to prepare of protein hydrolyzate of high nutritional value. The alkaline proteases are used in hydrolyzate production from various natural protein substrates. Keratinolytic activity of alkaline protease has also been exploited in the production of animal feed from waste feathers or keratin-containing materials.

Leather industry

The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and dangerous to humans. Thus, for environmental reasons, the bio-treatment of leather using an enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction, thus being eco-friendly. Alkaline proteases with combining of elastolytic and keratinolytic activity can be used in leather-processing industries. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced.

Photographic industry

Alkaline proteases play a crucial role in the bio-processing of used X-ray or photographic films for silver recovery. These waste films contain 1.5 – 2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Conventionally, this silver is recovered by burning the films, which causes undesirable environmental pollution. Furthermore, base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled. Alkaline protease from *B. subtilis* decomposed the gelatin layer within 30 min at 50 – 60°C and released the silver (Fujiwara et al., 1989).

Management of industrial and household waste

Proteases can digest proteinaceous waste and reduce the biological oxygen demand of aquatic systems. Recently, the use of alkaline protease in the management of wastes from various food-processing industries and household activities have been investigated and used for waste management. Alkaline protease from *B. subtilis* was used to manage feather waste from poultry slaughterhouses. Waste feathers are approximately 5% of the

body weight of poultry and are considered to be a high protein source for food and feed, provided their rigid keratin structure is completely destroyed.

Medical usage

Alkaline proteases are also used for developing products of medical importance. Kudrya and Simonenko (1994) found that the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses (Kudrya and Simonenko, 1994).

Detergent industry

Enzymes have long been of interest to the detergent industry for their ability to remove the proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies. Applications of detergent proteases have grown substantially and the largest application is in household laundry detergent formulations. The increased reliance of detergent manufacturers on enzyme technology is because of consumer recognizable cleaning benefits, the addition of completely new performance benefits, fabric restoration and an increased performance/cost ratio, because of the availability of more efficient enzymes and the industry trend toward reduced pricing.

Table 2.10 Commercial bacterial alkaline proteases, sources, applications and their industrial suppliers

Supplier	Product name	Microbial source	Applications
Novo Nordisk, Denmark	Alcalase	*B. licheniformis*	Detergent, silk degumming
	Savinase	*Bacillus* sp.	Detergent, textile
	Esperase	*B. lentus*	Detergent, food
	Biofeed pro	*B. licheniformis*	Feed
	Durazym	*Bacillus* sp.	Detergent
	Novozyme 471MP	*n.s.*	Photographic gelatin hydrolysis
	Novozyme 243	*B. licheniformis*	Denture cleaners
Genencor International, USA	Purafact	*B. lentus*	Detergent
	Primatan	Bacterial enzyme	Leather
Gist-Brocades, The Netherlands	Subtilisin	*B. alcalophilus*	Detergent
	Maxacal	*Bacillus* sp.	Detergent
	Maxatase	*Bacillus* sp.	Detergent
Solvay Enzymes, Germany	Opticlean	*B. alcalophilus*	Detergent
	Optimase	*B. licheniformis*	Detergent
	Maxapem	Protein engineered variant of *Bacillus* sp.	Detergent
	HT-proteolytic	*B. subtilis*	Alcohol, baking, brewing, feed, food, leather, photographic waste
Amano Pharmaceticals, Japan	Protease	*B. licheniformis*	Food, waste
	Proleather	*Bacillus* sp.	Food
	Collagenase	*Clostridium* sp.	Technical
	Amano protease S	*Bacillus* sp.	Food

n.s.; Not specified

Source: Gupta et al. (2002)

CHAPTER 3 MATERIALS AND METHODS

3.1. Raw Materials and Chemicals

3.1.1 Raw Materials

Shells and heads of marine Pacific white shrimp (*Litopenaeus vannamei*) were obtained from a seafood wholesaler in Samut Sakhon province, Thailand. Both bio-wastes were packed into an ice box for transportation and stored frozen at -20°C in the laboratory until used. Before proximate analysis, both shrimp bio-wastes were washed thoroughly with tap water and dried in a hot-air oven at 102°C for 12 h. For chitin and chitosan flake productions, shrimp shells were homogenized in a blender until small sized pieces (10 -20 mm) were obtained; these were then kept frozen until used.

3.1.2 Chemicals

1. Acetic acid
2. Chloroform
3. Methanol
4. Con. m-phosphoric acid
5. Con. hydrochloric acid
6. Sulfuric acid (H_2SO_4)
7. Glycerol
8. NaOH
9. Isopropanol
10. Hexane
11. Dimethyl acetamide
12. Lithium chloride (LiCl)
13. Copper (II) sulfate pentahydrate ($CuSO_4$. $5H_20$)
14. Sodium potassium Tartrate ($KNaC_4H_4O_6.4H_2O$)
15. Sodium chloride (NaCl)
16. Trichloroacetic acid ($C_2HCl_3O_2$)
17. Azocasein

18. Tris-base ($C_4H_{11}NO_3$)
19. Polyacrylamide $(C_3H_5NO)_n$
20. N,N'-Methylenebisacrylamide ($C_7H_{10}N_2O_2$)
21. Tetramethylethylenediamine (EDTA)
22. β-mercaptoethanol
23. Glycine
24. Bromophenol blue
25. Tyrosine
26. Sodium dihydrogen phosphate
27. Sodium dihydrogen phosphate (NaH_2PO_4)
28. Disodium hydrogen phosphate (Na_2HPO_4)
29. Potasium dihydrogen phosphate (KH_2PO_4)
30. Dipotasium hydrogen phosphate (K_2HPO_4)
31. Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)
32. Sodium carbonate (Na_2CO_3)
33. N-acetyl glucosamine

3.2 Equipment for Analysis

3.2.1 Glass Equipment

1. Beaker
2. Glass rod
3. Pipette
4. Burette
5. Glass funnel
6. Flask; 250 and 500 ml
7. Volumetric cylinder
8. Slide
9. Cover glass
10. Glass tube
11. Evaporator flask
12. Test tube

3.2.2 Machines

1. Digital balance 6 digits (Mettler Toledo, Thailand)
2. Laminar air flow (Astec Microflow Limited, Model ABS1200)
3. Microwave (Light Up Dial, Sharp, Model R 241)
4. Refrigerator (Expresso Cool, LG)
5. Freezer (Frezeer Cool, Sanyo)
6. Rotary vacuum evaporator (Buchi rotavapor R200, Switzerland)
7. Light microscope (Olympus, type CH-2, Olympus Optical Co., Ltd, Japan)
8. Electrophoresis (Power PAC 200, Bio-Rad)
9. Hot plate and Stirrer (IKA, Model C-MAG HS7, made in China)
10. Vortex mixture (Vortex 2, Scientific Industries, New York, USA)
11. UV-visible spectrophotometer (U-1800 spectrophotometer)
12. Digestion unit (Model K-424, Buchi)
13. Vortex mixture (Vortex genie-z of scientific industries, USA)
14. Autoclave (SA300VL, Japan).
15. Oven dry Unit (Carbolite)
16. Autopipette (100-1000µL), Autopipette (20-200µL), Autopipette (1 – 5 ml) and Autopipette (2-20µL) (Boeco, Germany)
17. Incubator (EcoCell 111, Germany)
18. Solvent extractor (Velp scientifica, Model SER 14-B)
19. Distillation unit (UDK 132, Semiautomatic distillation, Velp Scientifica)
20. Bio Fermentor (Volume 2 liter, Biostat-B, Biotech International)
21. pH Meter (Model 713, Metrohm)
22. Centrifuge (Avanty centrifuge, Model J26-XPI, Beckman Coulter)
23. Small centrifuge (IEC Centra-M2 centrifuge, made in USA)
24. Mini spin (Eppendorf)
25. Orbital shaker (Thermo Forma)
26. Shaking water bath (Model VS-1205SWL, Vision)
27. Digital camera (Sony Cybershot D D33021)
28. Microplate reader (Varian Cary 50 MPR, Varian, Inc., USA)
29. Rheometer (PHYSICA MCR 150)
30. Hunter Lab UltraScan XE Colorimeter (HunterLab, USA)

3.3 Methods

3.3.1 Shrimp Shell Preparation and Chemical Analysis

Shrimp bio-wastes; shrimp abdominal and head parts of a marine Pacific white shrimp *(Litopenaeus vannamei*) was washed thoroughly with tap water, dried in oven, and milled into powder. Total protein, lipid, and ash contents of the shrimp powder were determined by the standard methods followed AOAC (1995). Shrimp shells were used to prepared chitin and chitosan, whereas shrimp heads were considered as a source of growth supplements for microorganism.

3.3.2 Sources of Protease Producing Bacteria

Soil samples, from mangrove areas and coastline region in Bangkhuntien, Bangkok, were collected and used for isolation of proteolytic microorganisms. Sample characteristics were also recorded.

3.3.3 Screening and Isolation of Proteolytic Bacteria

Soil samples obtained from the section 3.3.2 were used to screen for the protease producing bacteria by using 1% skim milk medium, extracellular protease selective medium. A suitable dilution of soil suspension samples dissolved in 0.85% NaCl solution was dropped on protease detection agar and spread plate technique was done. The colony of extracellular protease producing bacteria which would be surrounded by a clearance zone on the protease selective agar after incubation at 37°C for 2 days were selected. All isolates were stored in 20% glycerol and kept in a freezer at -80^{o}C for long term storage and cultured on slant medium for immediate use. Proteolytic bacteria obtained from the screening were tested for the casein digestion efficacy on 1% skim milk agar to obtain the high potential of extracellular proteolytic bacteria. Proteolytic bacteria which showed a high ratio of clearance to bacterial colony (Ratio $\geq$ 5.0) were selected for further experiments.

3.3.4 Selection of Protease Producing Bacteria for Deproteinization of Shrimp Shells

The bacterial isolates obtained from the section 3.3.3 were tested for the efficacy of protease production in basal salt medium containing 2% shrimp shell powder (0.1% KH_2PO_4 and 0.05% $MgSO_4.7H_2O$). Protease activity was determined at 2 days of cultivation. Protease assay was determined by a modified method of Kunitz et al. (1947) by using casein as a substrate, (Appendix C). The bacterial isolate which gave the highest protease activity was selected for the process of deproteinization of shrimp shells in the further experiments.

3.3.5 Deproteinization of Shrimp Shells by the Selected Proteolytic Bacterial Isolate

The deproteinization of shrimp shells by the selected proteolytic bacteria obtained from the section 3.3.4 was performed in basal salt medium (0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$) with 3% (w/v) shrimp shells flakes. 1% (v/v) of the bacterial inoculums prepared in nutrient broth (NB) for overnight at 37^{o}C, 200 rpm was inoculated. During deproteinization process for 6 days, the number of bacterial cells, protease activity, pH, and soluble protein in the extraction liquid, as well as, the total protein, and ash contents of the hydrolyzed shrimp shells were determined. Protein content in shrimp shells was analyzed by a modified Biuret method as described by Gornall et al. (1949). Briefly, 100 mg dried materials were digested with 10 ml 0.5 M NaOH for 4 h at 40^{o}C and the mixture of alkaline solution was collected by centrifugation and used to estimate the protein concentration (Appendix B). The percent of deproteinization (%DP) was calculated using the following equation (3.1) (Rao et al., 2000):

$$\%\mathrm{DP} = \frac{\lfloor(P_O \times O) - (P_R \times R)\rfloor \times 100}{P_O \times O} \quad (3.1)$$

where P_O and P_R are the protein concentrations (%) of the shrimp shells before and after hydrolysis, respectively; while, O and R are the mass (g) of the initial sample and hydrolyzed residue on a dry weight basis, respectively.

3.3.6 Identification of the Selected Proteolytic Bacterial Isolate

The cell morphological and biochemical properties were used to identify the selected bacterial isolate. Phase contrast microscope was used to observe the bacterial structure. Molecular biological technique was used to determine 16S rRNA gene of the selected strain. 27F (5'-AGA GTT TGA TC(AC) TGG CTC AG-3') and 1525R (5'-AAA GGA GGT GAT CCA GCC – 3') primers were selected and used to amplify 16S rRNA gene. The nucleotide sequence of 16S rRNA gene of the selected isolate was determined by an ABI Prism 310 DNA Sequencer (PE Applied Biosystems, USA) with specific primers (T7 and SP6 primers), and were compared with the public sequence databases using Basic Local Alignment Search Tool (BLAST) of The National Center for Biotechnology Information (NCBI) to support the identification. The phylogenetic tree was constructed by using distance matrix method.

3.3.7 Characterization of Major Protease and Partial Protease Purification

3.3.7.1 Protease Production

1.0 ml of the starter culture in nutrient broth incubated overnight at 37^{o}C, 200 rpm was subsequently transferred to 50 ml basal salt medium pH 7.0 (0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_20$) containing 2% shrimp shell powder. After incubation for 24 h, 50 ml of old culture was transferred into bioreactor containing 1200 ml fresh medium. The condition of bioreactor was set at 160 rpm, 37^{o}C, and aeration at 2 nL / ml for 3 days. The number of bacterial cells and protease activity in the bioreactor were determined.

3.3.7.2 Characterization of Major Protease

3.3.7.2.1 The Effect of Temperature and pH on Protease Activity

The effects of temperature and pH on protease activity were investigated. To determine the optimum temperature, protease activity was measured by a modified method of Kunitz et al. (1947) with changing incubation temperatures at 20, 30, 37, 50, 60, and 70^{o}C, respectively, and 50 mM pH 7.0 Tris-HCl was used as buffer solutions. The pH

optimum of protease was also measured in 50 mM of Tris –HCl at difference pHs including pH 5, 6, 7, 8, 9, 10, 11, 12, and 13. Crude protease, at suitable dilution was pre-incubated at 30^{o}C for 6 h in50 mM sodium acetate (pH 4.0~5.5), 50 mM sodium phosphate (pH 6.0 ~7.0), 50 mM Tris–HCl (pH 7.5 ~ 9.0) and 50 mM Glycine - NaOH (pH 10 - 13) buffers to measure the pH stability. To test the thermostability of the protease, the crude protease was incubated at various temperatures ranging from 4 to 80°C for 6 h, and then cooled in ice-water. The residue activity was measured in % remained protease activity comparing to standard assay conditions.

3.3.7.2.2 Determination of Protease Type

To determine the type of major protease, the effects of enzyme inhibitors and activator including phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol were observed by pre-incubating cell free supernatants of 5.0, 10.0 and 15.0 mM of each inhibitor at 30^{o}C for 30 min prior to the measurement of the residual activities. The enzyme activity assayed in the absence of them was set at 100%.

3.3.7.3 Partial Protease Purification

3.3.7.3.1 Ammonium Sulfate Precipitation

Cell free supernatant was collected by centrifugation at 10,000 rpm for 20 min. The resultant mixture was precipitated overnight at 4^{o}C by addition of 608 g/L ammonium sulfate (90% Saturated). The following purification steps were done at 4^{o}C.

3.3.7.3.2 QAE TOYOPEAL Column (1x20 cm)

Column was pre – equilibrated with 0.02 M pH 8.0 sodium phosphate buffer (Buffer A). The resultant dialysate obtained above was loaded into column. The unabsorbed protein fractions having protease activity were fractioned and washed through column with buffer A. The flow rate was set at 1.0 ml/min. The protease fractions were kept and dialyzed overnight against ionized water containing 0.02% NaN_3. The resultant dialysate was concentrated by freeze dry.

3.3.7.3.3 Size Exclusion Chromatography on TOYOPEAL HW 50

A soluble protein (Section 3.3.7.3.2) was obtained by addition of small amount of 0.05 M sodium phosphate buffer (pH 7.0) containing 0.025 M NaCl and 0.02% NaN_3(Buffer B) and was then chromatographed on TOYOPEAL 55F 2.5 cm diameter by 60 cm at flow rate of 0.5 ml/min, pre – equilibrated with buffer B. The eluted fractions having activity were collected and dialyzed against ionized water. The resultant dialysate was concentrated by freeze dry.

3.3.7.3.4 Ion Exchange Chromatography on Ceramic Hydroxyapatite Type II

The dialyzate having activity was transferred into ceramic hydroxyapatite (Bio-Rad), particle size of 20 μm equilibrated with 0.001 M sodium phosphate buffer, pH 6.8 (Buffer C). Protease was separated from interfered materials by gradient solution (0.01-0.5 M Potassium phosphate buffer). Fractions having protease activity were collected and concentrated. The purity and size of protease were checked by 10% SDS – polyacrylamide gel electrophoresis.

3.3.7.3.5 Protein Determination

After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm. Protein content was determined by Lowry's method and bovine serum albumin as the standard (Appendix B).

3.3.7.3.6 Electrophoresis

The molecular weight of purified protease was determined by sodium dedocyl sulfate – polyacrylamide gel electrophoresis (10% PAGE). The standard protein using to calibration was purchased from Nacalai tesque (Broad range).

3.3.8 Sources of Acid Producing Bacteria

Acid producing bacteria were screened and isolated from the Thai traditional fermented sausages; Nham, purchased from supermarkets in Bangkok, Thailand.

3.3.9 Screening and Isolation of Acid Producing Bacteria

De Man Rogosa and Sharpe (MRS) medium was used for acid producing bacterial isolation. 1.0 g of Nham sample was added into 25 ml MRS broth. The acid producing bacteria was enriched by incubation at 37^{o}C for 48 h under static condition. Streak plate technique was done on MRS agar containing 0.5% $CaCO_3$. The bacterial colonies surrounded by clear zone were selected and purified. Preliminary identification was done by Gram reaction and catalase test. The bacterial isolates were maintained on MRS agar slants for immediate use and in 20% glycerol at -80^{o}C for stock culture.

3.3.10 Determination of Acid Production

Acid producing bacteria obtained from the section 3.3.9 was observed on acid production efficacy in MRS broth. The overnight culture of the acid producing bacterial isolates were adjusted their optical density (600 nm) to be 0.3 and used as inoculum at the concentration of 0.1% (v/v) for testing ability to produce acid in MRS broth. The pH, total titrable acidity (TTA) and bacterial cells in the broth culture were determined at 2 days (Appendix C). The isolate which showed the highest acid production was selected for decalcification of shrimp shells in the further experiment.

3.3.11 Decalcification of Shrimp Shells

3.3.11.1 Inoculum Preparation

The selected acid producing bacterial isolate was cultured in 100 ml of MRS broth overnight at 37^{o}C. The culture was centrifuged (Avanti J-26XPI Centrifuge, Beckman Coulter, USA) at 10,000 rpm for 10 min. The cell pellets were washed twice with 0.85 % NaCl, re-suspended with same solution (100 ml) and adjusted cell concentration to be at 10^6 CFU/ml and used as bacterial inoculum for shrimp shell fermentation.

3.3.11.2 The Effect of Glucose on Shrimp Shell Fermentation

The frozen shrimp shells were thawed in a refrigerator (4^{o}C) and homogenized with blender for 1 min. The effect of glucose on accelerating the demineralization of the shrimp shells was tested. 50 g of shrimp shells were mixed with 50 ml of sterile 2%

NaCl solution amended with glucose at the concentration of 10, 20, and 30% (w/w $_{\text{shrimp shell weight}}$) in 250 ml conical flasks. The fermentation was stated by incubation at 37^{o}C for 5 days under static condition. The bacterial inoculum was applied at 10% (v/v). After fermentation, solid residue was collected by filtration, washing with tap water and dried in oven at 102^{o}C for 12 h.

3.3.11.3 The Effect of Bacterial Loading on Shrimp Shell Fermentation

To determine the effect of bacterial loading on deproteinization and decalcification of shrimp shells, the optimum concentration of glucose from the previous experiment (Section 3.3.11.2) was used with varying the bacterial inoculum to be 10, 20, and 30% (v/v). The fermentation was started by incubation at 37^{o}C for 5 days under static condition. After fermentation, solid residue was collected by filtration, washing with tap water and dried in oven at 102^{o}C for 12 h. Solid residue was collected by filtration, washing with tap water and dried in oven at 102^{o}C for 12 h.

The pH, total titrable acidity (TTA) and bacterial cell concentration were determined every day until 5 days (Appendix B).Ash content in fermented shrimp shells was determined by heating sample in oven at 550^{o}C for 12 h (Appendix A). Total protein content in shrimp shells was determined according to the section 3.3.5. Decalcification (DC) and deproteinization (DP) were expressed as percentages and calculated using followed equation (3.1) where P_O and P_R are protein concentrations (%) before and after fermentation; while, O and R represent the mass (g) of original sample and fermented residue in dry weight basis. DC (%) was estimated with the same equation but replacing P_O and P_R with A_O and A_R which represent ash content in the original and fermented residue, respectively.

3.3.12 The Effect of Carbon Sources on Decalcification of Shrimp Shells

3.3.12.1 Inoculum Preparation

The inoculum for shrimp shell fermentation was prepared according to the section 3.3.11.1.

3.3.12.2 The Effect of Whey, Sucrose and Molasses on Shrimp Shell Fermentation

The fermentation was done by using different carbon sources with varying the concentration. For sucrose, sucrose at the concentrations of 10, 15, 20, and 30% (w/w $_{\text{shrimp shell weight}}$) were supplemented. Molasses was also used as carbon source for acid production. The effect of molasses on decalcification efficacy of shrimp shells was determined by varying the concentration of molasses at 10, 15, 20, 30, and 40% (w/w $_{\text{shrimp shell weight}}$). For whey, whey solutions at different concentrations of 50, 75, and 100% were supplemented for fermentation. The fermentation was started by incubation at 37^{o}C for 3 days under static condition. The pH, total titrable acidity (TTA) and bacterial cell concentration were determined every day until 3 days (Appendix C). Ash content in decalcified shrimp shells was determined. Total protein content in shrimp shells and decalcified shrimp shells was determined according to the section 3.3.5. Decalcification (DC) and deproteinization (DP) were expressed as percentages and calculated using followed equation (3.1).

3.3.13 Identification of the Selected Acid Producing Bacterial Isolate

The acid producing bacterium obtained from the section 3.3.10 was identified by using cell morphology and biochemical properties. The 16S rRNA gene of the selected isolates were amplified by PCR technique using 800R (18 mers; TAC CAG GGT ATC TAA TCC) and 518F (20 mers; CCA GCA GCC GCG GTA ATA CG) primers (Macrogen Inc, Seoul, Korea). The 16S rRNA gene was compared to the public sequence databases using Basic Local Alignment Search Tool (BLAST) of The National Center for Biotechnology Information (NCBI) for supporting the bacterial identification. The phylogenetic tree explaining relationship between these isolates and closed species was constructed by using distance matrix method.

3.3.14 Chitin Purification

3.3.14.1 Chemical Chitin Extraction

A mixture of 500 g of wet shrimp shells and 2,250 ml of 4% HCl was kept at room temperature for 4 h to eliminate inorganic components in the shells. The decalcified shrimp shells were then separated and washed several times with tap water. Deproteinization was performed by adding the dried decalcified shrimp shells into 5% NaOH at a ratio of 1:10, w/v. The mixture was shaken in a 90°C water bath for 12 h. Chitin flakes were obtained by washing the solid residue with tap water and drying overnight in an oven at 80^{o}C.

3.3.14.2 Preparation of Shrimp Head Extract Solution (SHES)

Shrimp heads from the section 3.3.1 were thawed in refrigerator (4^{o}C) and used to prepare shrimp head extract solution (SHES). SHES was prepared by boiling shrimp heads in deionized water. Briefly, shrimp heads were mixed with deionized water at a ratio of 1:2 (50%, w/v) and boiled for 30 min. SHES was obtained by filtering the solution through cotton to remove the sediment, and then adding deionized water to obtain the initial liquid volume before heat extraction.

3.3.14.3 Determination of *L. pentosus* L7 Growth in Various Culture Media Having SHES as a Major Component

L. pentosus L7 was cultured in de Man Rogosa and Sharpe medium (MRS), pH 6.6 (20 g glucose, 10 g peptone, 5 g beef extract, 5 g yeast extract, 5 g sodium acetate, 2 g triammonium citrate, 2 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4.4H_2O$, 1 g Tween 80, and 1,000 ml distilled water) and incubated at 37°C 24 h for use as an inoculum. The growth of *L. pentosus* L7 in the following culture media: (1) MRS, (2) SHES, (3) SHES plus 2% glucose, (4) SHES plus 2% NaCl, (5) SHES plus 2% glucose and NaCl, and (6) modified MRS medium (using SHES to replace peptone, beef extract, and yeast extract) was determined at 37°C using the spread plate technique on MRS agar.

3.3.14.4 Determination of *B. thuringiensis* SA Growth in SHES Compared to Nutrient Broth (NB) and Tryptic Soy Broth (TSB)

B. thuringiensis SA was cultured at 37°C in nutrient broth (NB), pH 7.0 (5 g peptone, 3 g beef extract, 3 g yeast extract, 5 g NaCl, and 1,000 ml distilled water) for 24 h and used as an inoculum. SHES was neutralized with 2 N NaOH to obtain pH of 7.0 ± 0.2 and sterilized at 121ºC for 15 min. The growth of *B. thuringiensis* SA in SHES was determined at 37°C using the spread plate technique on nutrient agar (NA) compared with the growth in NB and TSB.

3.3.14.5 Biological Chitin Purification

A 24 h culture of *L. pentosus* L7 in SHES plus 2% glucose was used as a starter in the decalcification step. Shrimp shell flakes (500 g) were mixed with 500 ml of starter (1:1, w/v) followed by 10% (w/w) glucose and 1% (w/w) salt in a 1,000 ml conical flask. Fermentation was performed at 37°C for 48 h; the decalcified shrimp shells were then separated by a sifter. The retentate was washed with tap water and dried in an oven overnight at 80^{o}C. For deproteinization of the decalcified shrimp shells, 10% (v/v) of 24 h culture of *B. thuringiensis* SA in SHES medium was added to a sterilized mineral solution (0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$) containing 3% (w/v) dried decalcified shrimp shells. The mixture was incubated at 37°C with agitation at 200 rpm for 72 h. The chitin was washed and dried overnight in an oven at 80^{o}C. Physical and chemical properties of chitin were examined.

Reducing sugar was determined by Somogyi-Nelson method (Appendix B). Ash content in decalcified shrimp shells was determined by heating sample in oven at 550^{o}C for 12 h (Appendix A). Total protein content in shrimp shells was determined according to the section 3.3.5. Decalcification (DC) and deproteinization (DP) were expressed as percentages and calculated using followed equation (3.1) where P_O and P_R are protein concentrations (%) before and after fermentation; while, *O* and *R* represent the mass (g) of original sample and fermented residue in dry weight basis. DC (%) was estimated with the same equation but replacing P_O and P_R with A_O and A_R which represent ash content in the original and fermented residue, respectively.

3.3.15 Determination of Chitin Quality

3.3.15 .1 Degree of Acetylation on Chitin

Degree of acetylation on chitin samples were determined by UV-VIS Spectroscopy methods described by Wu and Zivanovic (2008). The method was described below.

Establishing the calibration curve:

The calibration curve was made by plotting the first derivative UV values at 203 nm as a function of *N*-acetyl-D-glucosamine (GlcNAc) and D-(+)-glucosamine hydrochloride. Standard solutions of GlcNAc and GlcN were prepared in 0.85% phosphoric acid at concentrations of 0, 12.8; 25.6; 38.4; 51.2 and 64.0 μg/ml. 0.85% H_3PO_4 was used as the reference liquid (Appendix B).

Sample preparation and determination of DA:

Aliquots of 100 ± 10 mg chitin or chitosan were heated in 20 ml 85% phosphoric acid for 40 min at 60°C with constant stirring. After 40 min, when chitin/chitosan was completely dissolved, 1 ml clear solution was taken and diluted to 100 ml with deionized water. The dilution was necessary to get the chitin/chitosan concentration to the range detectable by a spectrophotometer. The diluted solutions were incubated at 60 °C for 2 h prior the UV measurement. Next the UV/Vis spectra were carried out in range 190 – 400 nm. 0.85% H_3PO_4 was used as the reference liquid. The degree of acetylation of chitin and chitosan samples was calculated as:

$$DA\ (\%) = \frac{\frac{m1}{203.21} \times 100}{\frac{m1}{203.21} + \frac{m2}{161.17}} \tag{3.2}$$

where: m1 is the mass of N-acetyl-glucosamine in 1 ml chitin/chitosan solution, calculated from the calibration curve by the corresponding λ = 203 nm, m2 is the mass of glucosamine in 1 ml chitin/chitosan solution, m2 = M - m1, M is the mass of chitin/chitosan in the 1 ml solution.

$$M = (M1 \times M3)/(M1 + M2), \quad (3.3)$$

where M1 is mass of solid chitin/chitosan sample taken for analysis (100 ± 10 mg), M2 is mass of 20 ml 85% phosphoric acid (34 g), M3 is mass of 1 ml chitosan solution in concentrated phosphoric acid (1.7 g).

3.3.15.2 Color Analysis

Value of L*, a* and b* were measured by Hunter Lab UltraScan XE Colorimeter (HunterLab, USA) with CIELAB color system. L* indicates lightness with scale ranging from 0 (back) to 100 (white). Positive and negative values in a* represent red and green, whereas positive and negative values in b* represent yellow and blue, respectively. Whiteness index (WI) was calculated based on the following equation (3.4) described by Hsu et al. (2003)**:**

$$WI=100-[(100-L^*)^2+a^{*2}+b^{*2}]^{1/2} \quad (3.4)$$

3.3.15 .3 Shear Viscosity Analyses

Chitin samples were dipped into 50% ethanol at 45^{o}C to remove fat content. The chitin was crushed into powder. N,N-dimethylacetamide (DMA) (99.5%, anhydrous, UNILAB, Ajax Finechem, Australia) containing 5% (w/v) lithium chloride (LiCl) (98.0%, anhydrous, LABA Chemie, India) were used to prepare 0.1% (w/v) chitin solution. The mixture was stirred for 120 h at room temperature. The shear viscosity was measured by Rheometer (PHYSICA MCR 150). A cone and plate geometry with a cone angle of 2.0^o and radius of 24.975 mm was used for the measurements, and the gap size was 0.49 micron. The shear rates ranged from 0.1 to 1000 sec^{-1} with 5 data points per log cycle. The experiments were conducted at 25^{o}C.

3.3.16 Chitosan Production

3.3.16.1 Chitosan Production from Chemically Processed Chitin

Chitin obtained from the section 3.3.14.1 was used to prepared chitosan. 1 g Chitin was added into bottom containing 25 ml 50% NaOH and closed with aluminum seal. Deacetylation was done at 121^{o}C (Temperature of hot-air oven) by increasing reaction times from 2 h to 6 h. The degree deacetylation (%DD) was determined according to the section 3.3.15.1.

3.3.16.2 Chitosan Production from Biologically Purified Chitin

Biologically purified chitin obtained from the section 3.3.14.5 was used to prepared chitosan. Chitosan was prepared according to the section 3.3.16.1.

3.3.17 Quality Analysis of Chitosan Samples

Degree acetylation and color properties of prepared chitosan were determined according to section 3.3.15.1 and 3.3.15.2. For shear viscosity analysis, the chitosan samples were crushed into powder and dissolved in 1% acetic acid to prepare 0.1% (w/v) chitosan solution. The mixture was stirred for overnight at room temperature. The shear viscosity was measured by Rheometer (PHYSICA MCR 150). A cone and plate geometry with a cone angle of 2.0^{o} and radius of 24.975 mm was used for the measurements, and the gap size was 0.49 micron. The shear rates ranged from 0.1 to 100 sec^{-1} with 5 data points per log cycle. The experiments were conducted at 20^{o}C.

CHAPTER 4 RESULTS

4.1 The Chemical Composition of Shrimp Shells and Heads

In shrimp processing, the shrimp bio-wastes are inedible parts of shrimp including head and abdominal portions removed before shrimp is cooked and packed to sell in the global market. Approximately 40% of total production value becomes wastes from the processing (Venugopal, 2009). In this study, shrimp bio-wastes were obtained from sea food industry in Samut Sakhon province, Thailand. Head and abdominal portions were dried in oven at 80^{o}C for 24 h and made to powder by homogenizer for determination of chemical compositions. Table 4.1 shows the composition of shrimp shells and heads. The shrimp shells had less protein and lipid than shrimp heads; therefore, abdominal parts were a good raw material for chitin preparation, whereas shrimp heads contained high protein and lipid contents indicating that they were a good source for animal feed production, growth supplements for microorganisms, or substrate to induce proteolytic enzyme.

Table 4.1 Chemical properties of shrimp heads and shells

Components (%, w/w)	**Shrimp shells (Abdominal part)**	**Shrimp heads**
Moisture	79.96 ± 0.28	86.13 ± 2.19
TKN	7.56 ± 0.35	9.06 ± 0.12
Crude protein	25.92 ± 2.03	40.74 ± 2.66
Lipid	2.37 ± 0.95	13.87 ± 1.12
Ash	27.92 ± 0.94	22.21 ± 0.61
Chitin[a]	43.79	23.18

[a]; by calculation

Each value is expressed as mean ± s.d. (n = 3)

(A)

(B)

Figure 4.1 Shrimp shells (A) and heads (B)

4.2 Sources of Protease Producing Bacteria

The soil samples used in this study for isolation of protease producing bacteria were collected from mangrove areas and coastline regions in Bangkhuntien, Bangkok, Thailand. The samples properties are recorded and shown in the Table 4.2.

Table 4.2 Soil sample characteristics for isolation of protease producing bacteria

Samples No.	Type of samples	Place	Sample Characteristic	Figures
1	Soil from coastline	Bangkhuntien	Sand with soil	
2	Soil from mangrove area	Bangkhuntien	Dark soil	
3	Soil from mangrove area	Bangkhuntien	Dark soil	

Table 4.2 Soil sample characteristics for isolation of protease producing bacteria (Cont.)

Samples No.	Type of samples	Place	Sample Characteristic	Figures
4	Soil from mangrove area	Bangkhuntien	Dark soil	
5	Soil from mangrove area	Bangkhuntien	Dark soil	
6	Soil from coastline	Bangkhuntien	Sand with soil	

The 6 soil samples were collected from mangrove and coastline areas which they should contain protease producing bacteria. Mangroves play a major role as a protector of the coast against erosion due to wind, waves, water currents, and protect coral reefs. They are also known to absorb pollutants. Mangroves area is habitats of various kinds of sea animals and returns nutrients to the marine food webs and provides spawning grounds to a variety of fish and shellfish. From above reasons, this area accumulates the nutrient from digestion of crabs and shrimps; therefore, mangrove area is considered as a good sample source for isolation of proteolytic bacteria to decompose protein in shrimp shells.

4.3 Screening and Isolation of Proteolytic Bacteria

Protease producing bacteria were isolated from soil of mangrove and coastline region in Bangkhuntien area by using the rich protein culture medium; 1% skim milk agar is known as non - specific protease substrate to isolate extracellular proteolytic microorganisms. A suitable dilution of soil suspension samples dissolved in 0.85% NaCl solution was dropped on protease detection agar and the spread plate technique was done. After 2 days of incubation at 37^{o}C, the potential protease producing strains were selected based on the zone of clearance surround the colony. From the screening, 14 bacterial isolates were obtained from 6 soil samples (Table 4.3). The primary identification was done to classify those bacteria. There were two major kinds of bacteria obtained from soil samples including Gram positive spore forming bacteria (8 isolates) and Gram negative none spore forming bacteria (6 isolates). Gram positive spore forming bacteria included bacterial isolates RA1, RA3, RA4, RA7, RA8 and RA10, whereas bacterial isolates SE1, SE2, RA2, RA5, RA6 and RA9 were pre-primary classified in the member of Gram negative none spore forming bacteria. Those bacteria obtained from the screening were investigated for the casein digestion efficacy on 1% skim milk agar to obtain the high potential of extracellular proteolytic activity. The results showed that 6 isolate including SE1, RA1, RA2, RA5, SA and P1 gave high ratio of clearance to bacterial colony (Ratio $\geq$ 5.0). They were selected for the further experiments. Table 4.4 shows ratio of clear zone to bacterial colony after incubation at 37^{o}C for 24 and 48 h. The bacterial colonies of 6 selected isolates growing on 1% skim milk agar are shown in Figure 4.2.

Table 4.3 The proteolytic bacteria isolated from mangrove area and coastline in Bangkhuntien

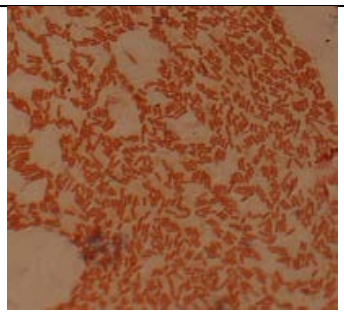
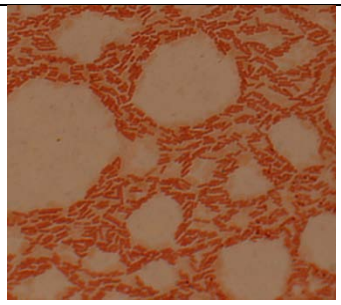
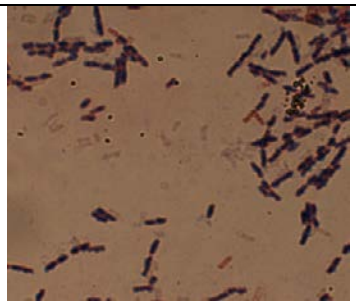
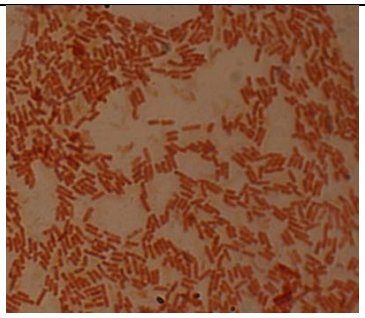
Samples No.	**Bacterial Isolates**	**Colony and cell morphologies**	**Figures**
1	SE1	Colony: white, circular, convex Cell: Gram negative, rod bacteria, no spore formulation	
	SE2	Colony: white, circular, convex Cell: Gram negative, rod bacteria, no spore formulation	
2	RA1	Colony: white, irregular, flat Cell: Gram positive, rod, spore forming bacteria	
	RA2	Colony: white, circular, convex Cell: Gram negative, rod bacteria, no spore formulation	

Table 4.3 The proteolytic bacteria isolated from mangrove area and coastline in Bangkhuntien (Cont.)

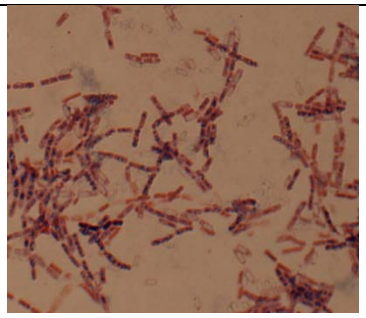
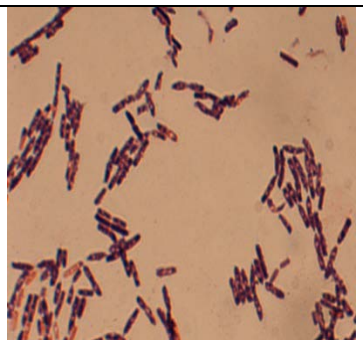
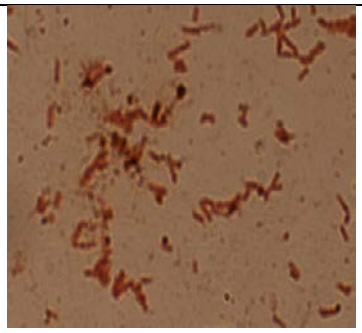
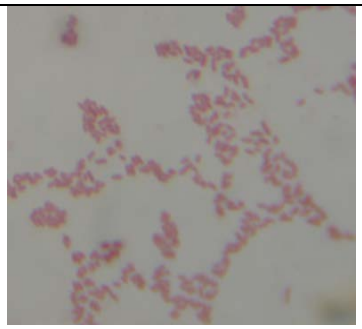
Samples No.	Bacterial Isolates	Colony and cell morphologies	Figures
2	RA3	Colony: white, irregular, flat Cell: Gram positive, rod, spore forming bacteria	
3	RA4	Colony: white, circular, flat Cell: Gram positive, rod, spore forming bacteria	
	RA5	Colony: yellow, circular, convex Cell: Gram negative, rod bacteria, no spore formulation	
4	RA6	Colony: white, circular, convex Cell: Gram negative, rod bacteria, no spore formulation	

Table 4.3 The proteolytic bacteria isolated from mangrove area and coastline in Bangkhuntien (Cont.)

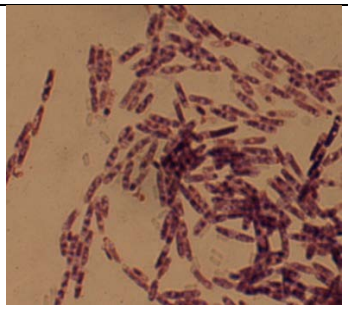
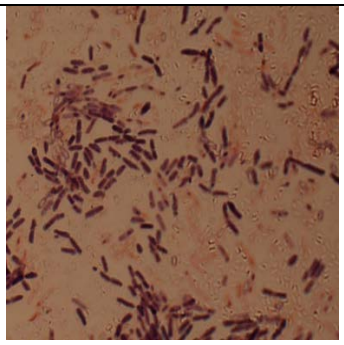
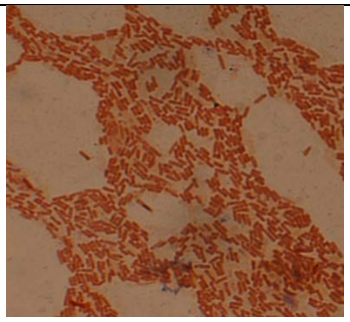
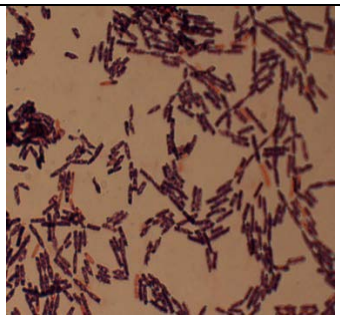
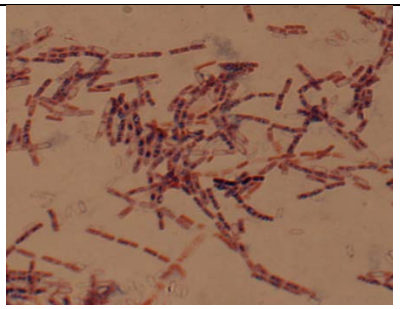
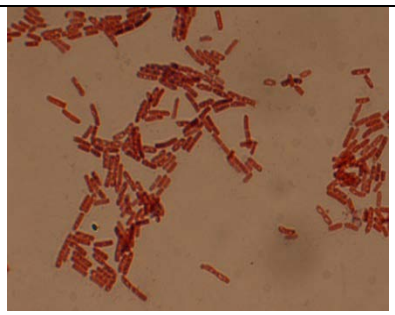
Samples No.	Bacterial Isolates	Colony and cell morphologies	Figures
4	RA7	Colony: white, circular, flat Cell: Gram positive, rod, spore forming bacteria	
5	RA8	Colony: white, circular, flat Cell: Gram positive, rod, spore forming bacteria	
	RA9	Colony: white, circular, convex Cell: Gram negative, rod bacteria, no spore formulation	
	RA10	Colony: white, circular, flat Cell: Gram positive, rod, spore forming bacteria	

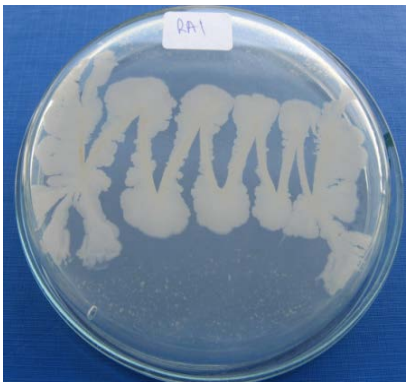
Table 4.3 The proteolytic bacteria isolated from mangrove area and coastline in Bangkhuntien (Cont.)

Samples No.	Bacterial Isolates	Colony and cell morphologies	Figure
6	SA	Colony: white, circular, flat Cell: Gram positive, rod, spore forming bacteria	
	P1	Colony: white, circular, flat Cell: Gram positive, rod, spore forming bacteria	

According to clear zone around the colony, 6 strains showed high ratios of clear zone to colony size on skim milk agar indicating that they could produce extracellular protease when growing on the rich protein medium (Table 4.4). However, these results do not guarantee that bacteria isolated by skim milk selective medium can decompose protein in shrimp shells because of protein sources in shrimp waste and skim milk are difference. To solve this problem, those bacteria (Isolate SE1, RA1, RA2, RA5, SA and P1) were cultured in basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$) containing 2% shrimp shell powder as sole of nitrogen and carbon source. The effective strains which showed the highest proteolytic activity were selected and used for deproteinization of shrimp shells in the further experiment.

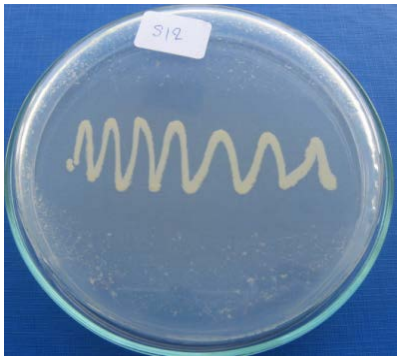
Table 4.4 Ratios of clear zone to bacterial colony after incubation at 37^{o}C for 24 and 48 h

Strains	**Ratio (size of clear zone / size of colony)**	
	24 h	**48 h**
SE1	4.2	6
SE2	3.01	4.03
RA1	3.91	6.04
RA2	4.75	6.25
RA3	3.21	4.23
RA4	2.56	3.98
RA5	4.38	6.25
RA6	2.33	4.64
RA7	3.01	3.98
RA8	2.98	3.87
RA9	2.91	4.18
RA10	3.03	4.09
SA	3.91	5.87
P1	5.38	5.18

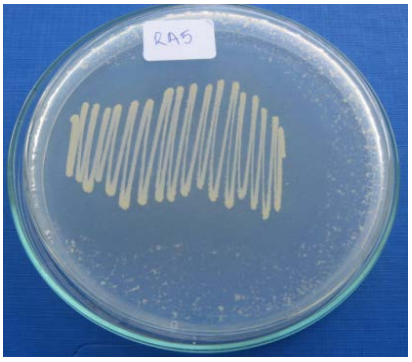


(A) RA1

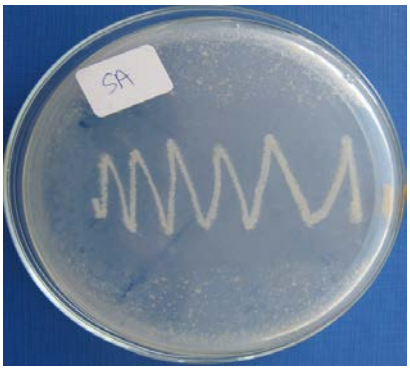
(B) RA2



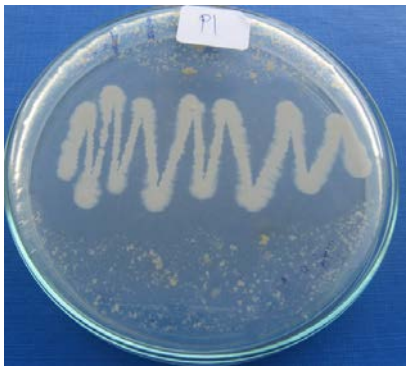
(C) SE1



(D) RA5



(E) SA



(F) P1

Figure 4.2 Protease producing strains cultured on skim milk agar after incubation at 37^{o}C for 2 days

4.4 Selection of Protease Producing Bacteria for Deproteinization of Shrimp Shells

The selected 6 strains of protease producing bacteria were investigated for their protease activities in basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$) containing 2% shrimp shell powder. The results are shown in Table 4.5. After incubation for 2 days at 37^oC, protease activity was determined by using modification of Kunitz's method (1947). Supernatant was used as a crude enzyme to measure extracellular protease activity. Among them, isolate SA gave the highest protease activity indicating that bacterial isolate SA could use nitrogen and carbon from shrimp shells for growth. Protease activity of isolate SA after culturing in protease producing medium for 2 days was 45 ± 8.9 units/ml. In other hands, all isolates showed a high protease activity on skim milk agar, but isolate SA could only show high extracellular protease activity. This point indicated the inducing substrate had an effect on protease production and activity. The isolate SA could grow in the basal salt medium which had shrimp shell flakes as the sole carbon and nitrogen sources, and produce the high amount of protease specific for protein in shrimp shells, the isolate SA could be a good candidate for deproteinization of the shrimp shells by biological method. Therefore, the isolate SA was used as the effective strain to deproteinize shrimp waste.

Table 4.5 Protease activities of 6 different stains after incubated at 37^oC for 2 days

Strains	Protease activity (units/ml)
SE1	23 ± 9.9
RA1	22 ± 3.14
RA2	14 ± 5.8
RA5	26 ± 1.1
SA	45 ± 8.9
P1	10 ± 2.6

Each value is expressed as mean ± s.d. (n = 3)

4.5 Deproteinization of Shrimp Shells by the Selected Proteolytic Bacterial Isolate

The deproteinization of the shrimp shells by the selected proteolytic bacterium (Isolate SA) was performed in basal salt medium with 3% (w/v) shrimp shell flakes. The bacterial growth, protease activity, pH, soluble protein, and deproteinization efficacy in the extracting liquid and deproteinized materials during the deproteinization were demonstrated in Figures 4.3 and 4.4. The maximum protease activity of 10.75 units/ml was detected in 3 days. From the initial bacterial cell concentration at 6 log CFU/ml, the culture reached the maximum growth at about 8 log CFU/ml in one day and became stable at that level throughout the experiment (Figure 4.3). During the deproteinization, pH value was increased from 7.2 to 8.9 which could be explained by the release of ammonia by protein degradation process (Figure 4.4). The soluble protein in the culture supernatant increased from 1250 µg/ml to the maximum at 2250 µg/ml in the day 2 of the incubation. From the beginning, the deproteinization increased gradually and reached the maximum at the day 4 with about 90.33% deproteinization (Figure 4.4).

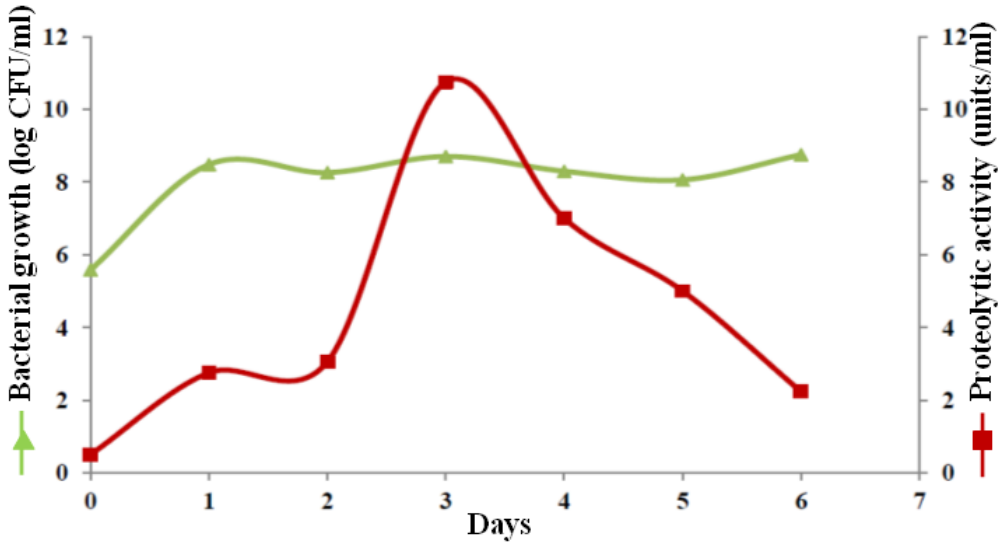


Figure 4.3 Growth and protease activity of the isolate SA in basal salt medium with 3%w/v of shrimp shells

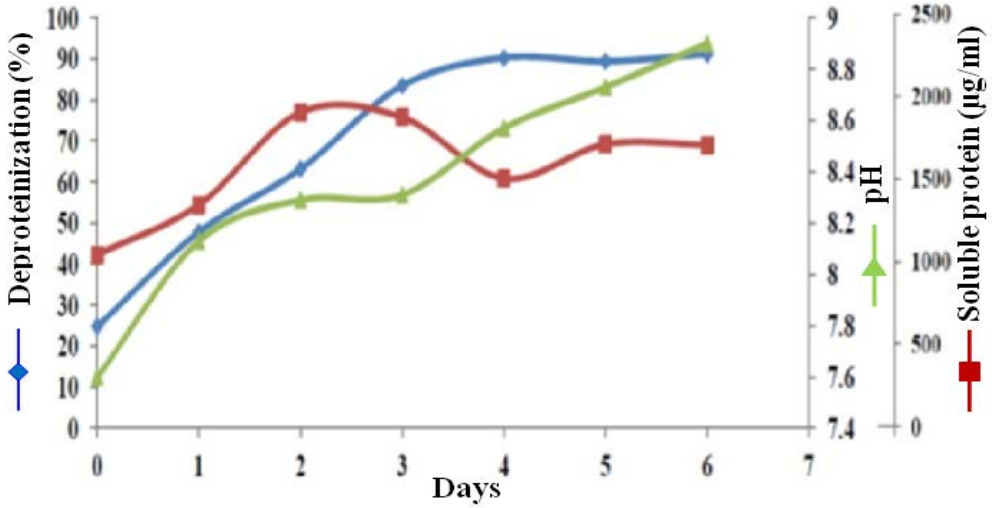


Figure 4.4 Changing in pH, amount of soluble protein, and % deproteinization during deproteinization by the isolate SA in basal salt medium with 3% (w/v) of shrimp shells

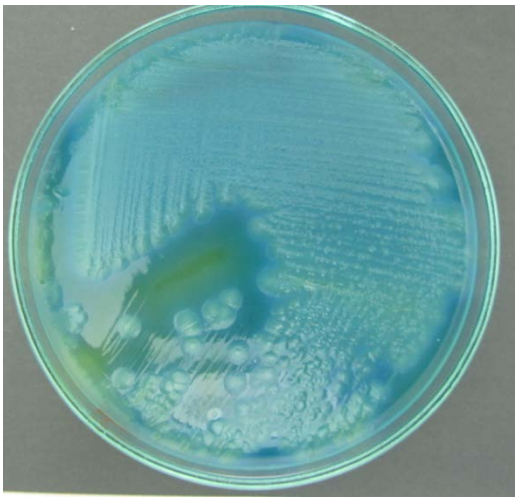
At the end of process, the residual crude protein and calcium in deproteinized shrimp shells were 5.78% and 33.47%, respectively. The crude protein in shrimp shells was decreased from 25.92% to 5.78% indicating that a deproteinization of shrimp shells by isolate SA application is possible to replace alkaline extraction in deproteinization step. 2% to 3% shrimp shell flakes were appropriate amount of shrimp shells for deprotinization. The excessive amount of shrimp shells (≥4%) did not stimulate protease activity. In the other hands, 4% shrimp shells decreased proteolytic activity due to high amount of soluble nutrients in liquid of culture medium (Table 4.6). This result indicated 3% shrimp shells were considered as the maximum shrimp shells for completely deproteinization by bacterial isolate SA.

Table 4.6 The residual crude protein in deproteinized shrimp shells and protease activity in culture supernatants

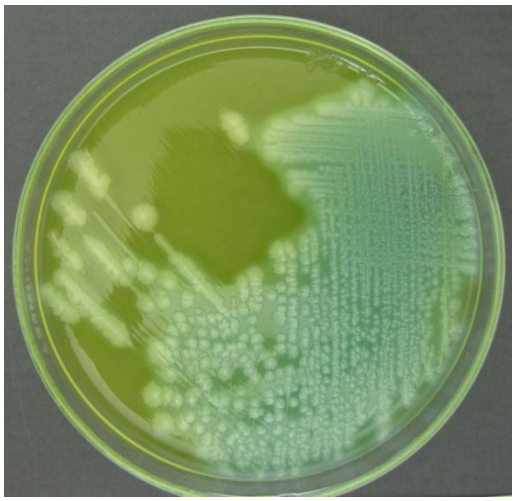
Shrimp shells (%)	Deproteinization time (days)	Protease activity (units/ml)	Residual crude protein (%, w/w)
2%	2	10.52	14.53
	3	15.21	5.64
	4	9.11	5.07
3%	2	3.45	16.21
	3	10.75	5.15
	4	6.23	5.78
4%	2	3.64	19.48
	3	10.11	9.23
	4	5.25	9.15

4.6 Identification of the Selected Proteolytic Bacterial Isolate

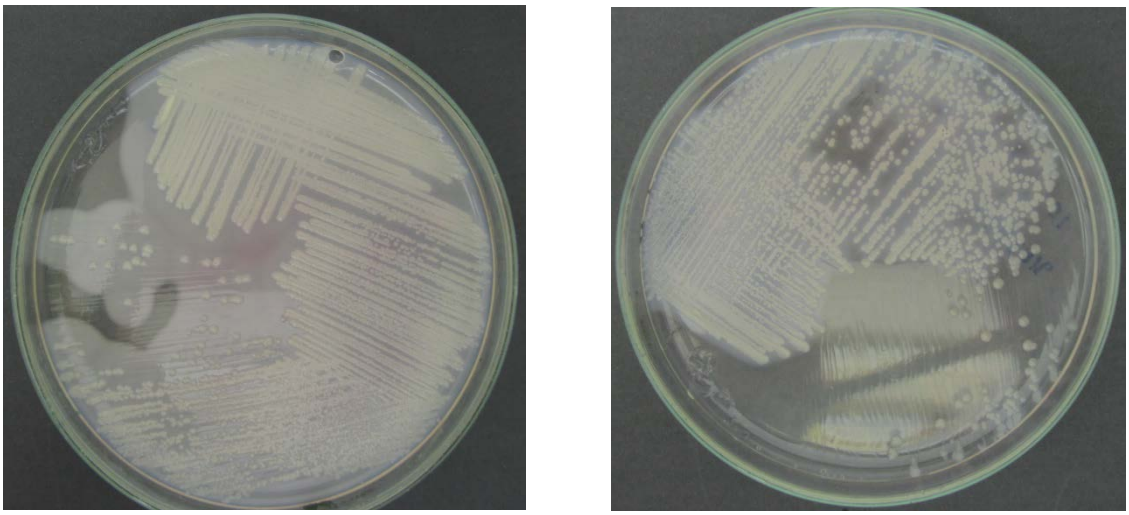
The cell morphological and biochemical properties were used to identify the selected bacterial isolate SA. The results revealed that isolate SA is Gram positive, rod shape and spore forming bacterium. Figure 4.6 shows isolate SA under light microscope. The isolate SA could grow on *Bacillus cereus* selective agar and lecithinase agar. Both media are commonly used for screening and detection of pathogenic *B. cereus* in foods and environments. In the formulation of *B. cereus* selective agar, a peptone level of 0.1% and the addition of sodium pyruvate can improve egg yolk precipitation and enhance spore formation. Bromothymol blue is added as a pH indicator to detect mannitol utilization. The medium is made for selective by addition of Polymyxin B. The primary diagnostic features of the medium are the colonial appearance, precipitation of hydrolysed lecithin and the failure of *B. cereus* to utilize mannitol. The typical colonies of *B. cereus* are recognized, about 5 mm in diameter. The colonies of *B. cereus* on *B. cereus* selective agar were peacock blue color surrounded by a good egg yolk precipitate of the same color. These features distinguish *B. cereus* from other *Bacillus* spp., except *Bacillus thuringiensis* (Figure 4.5). *B. cereus* is a pathogenic bacterium, often encountered in meat and poultry products. This pathogen is a usually strong positive producer of lecithinase enzyme, strongly hemolytic on sheep blood agar, and is actively motile. Other lecithinase positive or weakly positive cultures may be *B. thuringiensis* and *B. anthracis*.



(A)



(B)



(C) (D)

Figure 4.5 *B. cereus* and isolate SA cultured on *B. cereus* selective agar and lecithinase agar after incubation at 37^{o}C for 24 h; *B. cereus* (A) and isolate SA (B) cultured on *B. cereus* selective agar, *B. cereus* (C) and isolate SA (D) cultured on lecithinase agar

After incubation 37^{o}C for 24 h, isolate SA could grow on *B. cereus* selective agar. However, this isolate showed weak positive in producer of lecithinase (Figure 4.5). The isolate SA could be classified as *B. cereus*. Some biochemical characteristics were tested for identification of isolate SA (Table 4.7). The results showed that isolate SA gave similar biochemical characteristics to *Bacillus cereus*. For observation of spore formation, isolate SA was re-streaked on nutrient agar containing manganese (Mn) which is often used for promoting sporulation in *Bacillus* species (5 g peptone, 3 g beef extract, 0.03 g Manganese sulphate, 15 g bacterial agar and 1000 ml distilled water) and incubated at room temperature for 5 days. By phase contrast microscope, parasporal crystal body inside SA cell during sporulation state after incubated on sporulation agar for 2 days was observed (Figure 4.7). The present of parasporal crystal body inside the SA cell confirmed that the isolate SA was member of *B. thuringiensis*.

Table 4.7 Biochemical characteristics of *Bacillus cereus* and isolate SA

Feature	*B. cereus*	Isolate SA
Gram reaction	+	+
Catalase	+	+
Sporulation	+	+
Motility	+	+
Reduction of nitrate	+	+
Starch	+	+
Lysozyme-resistant	+	+
Egg yolk reaction	+	+
Glucose fermentation	+	+
VP reaction	+	+
Mannitol fermentation	–	–
Special characteristic	N.D.	Endotoxin crystals

N.D.; not detected

Base on 16S rRNA gene identification, full length of 16S rRNA gene was amplified by 27F (22 mers, 5'-AGA GTT TGA TC(AC) TGG CTC AG-3') and 1525R (18 mers, 5'-AAA GGA GGT GAT CCA GCC – 3') primers. A total 30 cycles of PCR condition to amplify 16S rRNA gene was pre-denaturation at 94^{o}C for 4 min, denaturation at 94^{o}C for 30 sec, primer annealing at 50^{o}C for 1 min, primer extension at 72^{o}C for 2 min and post-extension at 72^{o}C for 5 min. The direct sequencing cannot sequence the full length of 16S rRNA gene. The amplification products were purified with a gel excision kit (Promega, Inc., Madison, USA) and were cloned using pGEM-T Easy vector (Promega, Inc., Madison, USA). After sequencing, 1542 bases 16S rRNA gene of isolate SA was determined. The BLAST result is shown in Table 4.8. The use of full length of 16S rRNA gene revealed that the isolate SA was similar to *B. cereus* ATCC 10987 with 99% identity (Table 4.8). Phylogenetic tree of the isolate SA based on the nucleotide sequence of 16S rRNA gene is shown in Figure 4.8. Now a day, the use of 16S rRNA gene cannot identify at species level of *B. cereus* group (Including 4 species; *B. cereus, B. thuringiensis, B. mycoides,* and *B. anthracis*) due to the present of the high percentage of sequence similarity between closely related species in 16S rRNA gene (Ash et al., 1991; Christensen et al., 1998; Martı´nez-Murcia et al., 1992). The present

of parasporal crystal body in cell of *B. thuringiensis* during sporulation state was used as a significant key to classify *B. thuringiensis* from *B. cereus*. This result supported that 16S rRNA gene is not a good genetic guild to identify isolate SA.

In the other hands, Gyr B gene, a type II DNA topoisomerase, is often used to identify and reconstruct a phylogenetic tree of various species. Gyr B gene is protein-encoding gene which differs from 16S rRNA gene (Protein-unencoding gene). 16S rRNA gene transcribe to RNA which is a part composition of ribosomal protein. Gyr B gene was used to identify and reconstruct genetic evaluation in several families such as *Pseudomonas* (Yamamoto and Harayama, 1995; Yamamoto and Harayama, 1996; Yamamoto and Harayama, 1998) *Mycobacterium* (Kasai et al., 2000), *Salmonella*, *Shigella* and *Escherichia coli* (Fukushima et al., 2002), *Aeromonas* (Yanez et al., 2003), *Bacillus anthracis–cereus–thuringiensis* group (La Duc et al., 2004). Yamamoto and Harayama (1995) revealed that Gyr B gene is distributed universally among bacterial species and the rate of molecular evolution inferred from Gyr B gene sequences is faster than that inferred from 16S rRNA gene. Therefore, Gyr B gene was also used in this experiment.

For amplification of Gyr B gene sequence of isolate SA, GyrF (20 mers, 5'-TCT GGT GGT TTR CAY GGT GT-3') and GyrR (23 mers, 5'-GTY GTY TCC CAH AGC TGA GTT GG- 3') were generated for this experiment. PCR condition for 30 cycles was pre-denaturation at 94^{o}C for 4 min, denaturation at 94^{o}C for 30 sec, primer annealing at 50^{o}C for 2 min, primer extension at 72^{o}C for 2 min and post-extension at 72^{o}C for 5 min. The amplification products were purified with a gel excision kit (Promega, Inc., Madison, USA) and were cloned using pGEM-T Easy vector (Promega, Inc., Madison, USA). The nucleotides on sequences were determined by specific primer (T7 and SP6 primers). After sequencing, 1425 bases Gyr B gene of isolate SA was determined by this generated primers. The BLAST result revealed that the isolate SA is similar to *B. thuringiensis* serovar poloniensis strain IEBC-T54 001 with 98% identity (Table 4.9). According to previous results, Gyr B gene should be a suitable genetic marker to classify *B. cereus* group. Base on Gyr B gene and the present of parasporal crystal body inside SA cell, isolate SA was classify as *B. thuringiensis* strain SA. The phylogenetic tree explaining relationship between *B. thuringiensis* strain SA and closed species was constructed (Figure 4.10).

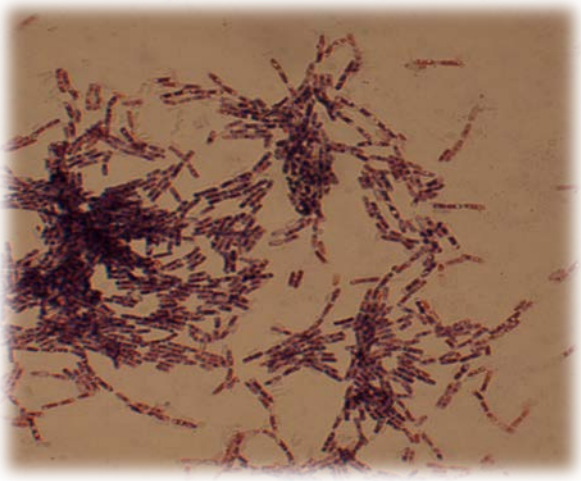


Figure 4.6 Gram stain of the isolate SA (100×)

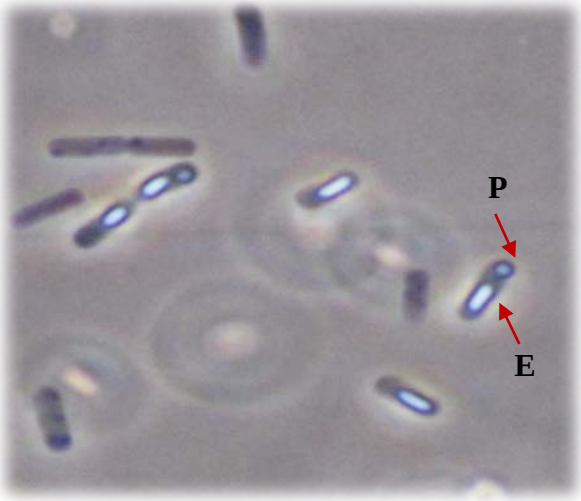


Figure 4.7 The proteolytic bacterial isolate SA under phase contrast microscope (1000×); (P) parasporal crystal body and (E) endospore

Table 4.8 BLAST result of 16S rRNA gene of isolate SA

Accession No.	Strains	Score	% Identity
CP001177.1	*Bacillus cereus* AH187	2832	99
AE017194.1	*Bacillus cereus* ATCC 10987	2832	99
CP001407.1	*Bacillus cereus* 03BB102	2826	99
CP000227.1	*Bacillus cereus* Q1	2826	99
CP001283.1	*Bacillus cereus* AH820	2826	99
CP000485.1	*Bacillus thuringiensis* Al Hakan	2826	99
GU566355.1	*Bacillus* sp. D12(2010)	2820	99
CP001598.1	*Bacillus anthracis* A0248	2820	99

Table 4.9 BLAST result of Gyr B gene of isolate SA

Accession No.	Strains	Score	% Identity
EF210281.1	*B. thuringiensis* serovar poloniensis strain IEBC-T54 001	2542	98
AE017194.1	*B. cereus* ATCC 10987	2536	98
CP001177.1	*B. cereus* AH187	2503	98
EF210249.1	*B. thuringiensis* serovar finitimus strain BGSC 4B2	2497	98
CP000227.1	*B. cereus* Q1	2492	98
EF210248.1	*B. thuringiensis* serovar finitimus strain BGSC 4B1	2492	98
EU761188.1	*B. thuringiensis* serovar pondicheriensis strain IEBC-T20A001	2303	95

AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGC
AAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACG
GGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAA
ACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAA
GGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGG
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TAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTA
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TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAG
TTGCCATCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACA
CACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCT
AATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACAT
GAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCG
AAGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATGA
TTGGGGTGAAGTCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCA
CCTCCT

(A) 1542 base of 16S rRNA gene of isolate SA

TCTGGTGGTTTGCATGGTGTTGGGGCATCTGTAGTAAATGCCCTATCAACAG
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CAGGAACGATAACTCGATTTAAACCAGATCCAGAAATTTTTCAGGAAACAA
CAGTATACGAATTTGATACGCTCGCAACTCGTATGCGTGAATTAGCATTTTT
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TTTTAGCTGAATTACCAGCTCAACCTAAGCCTGGTATCCAACGTTACAAAGG
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(B) 1523 bases Gry B gene of isolate SA

Figure 4.8 DNA sequences of 16S rRNA (A) and Gyr B (B) genes

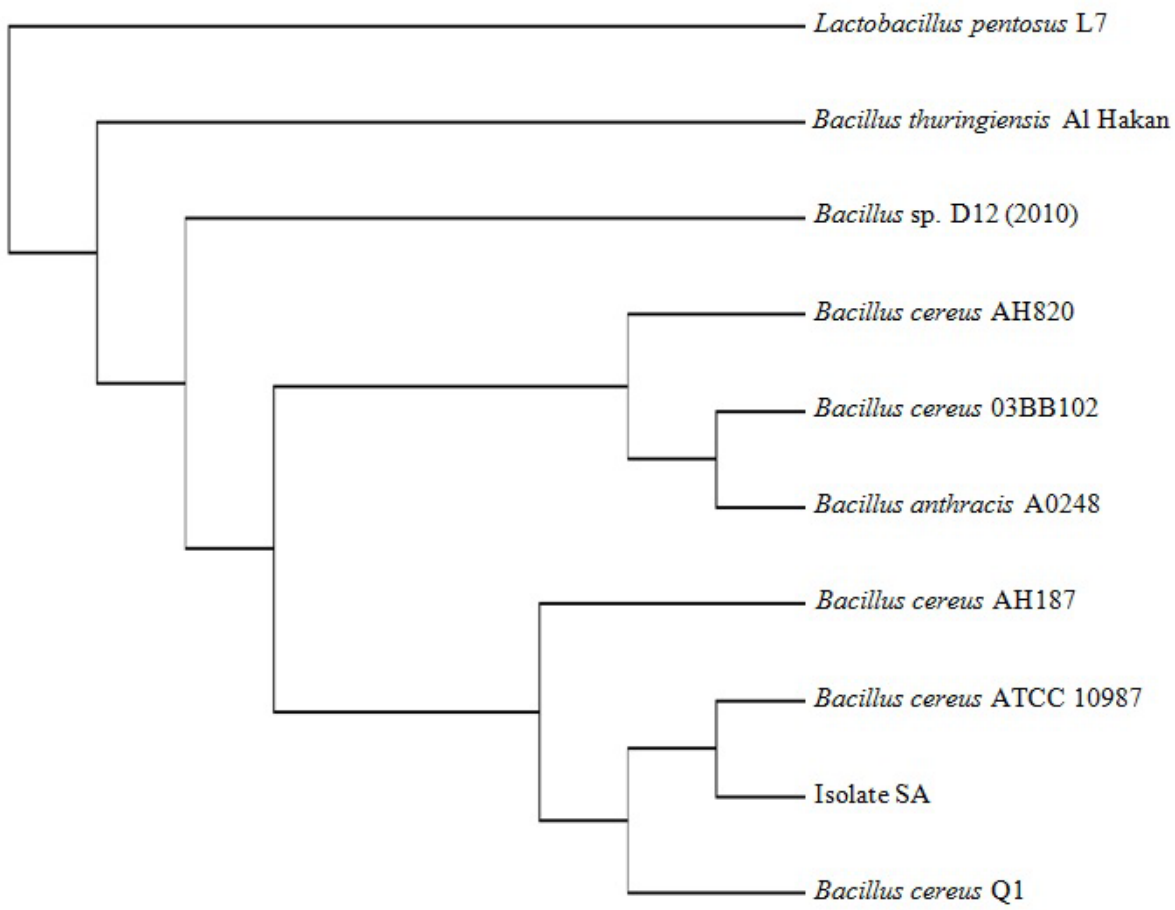


Figure 4.9 Phylogenetic tree of the isolate SA based on the nucleotide sequence of 16S rRNA gene

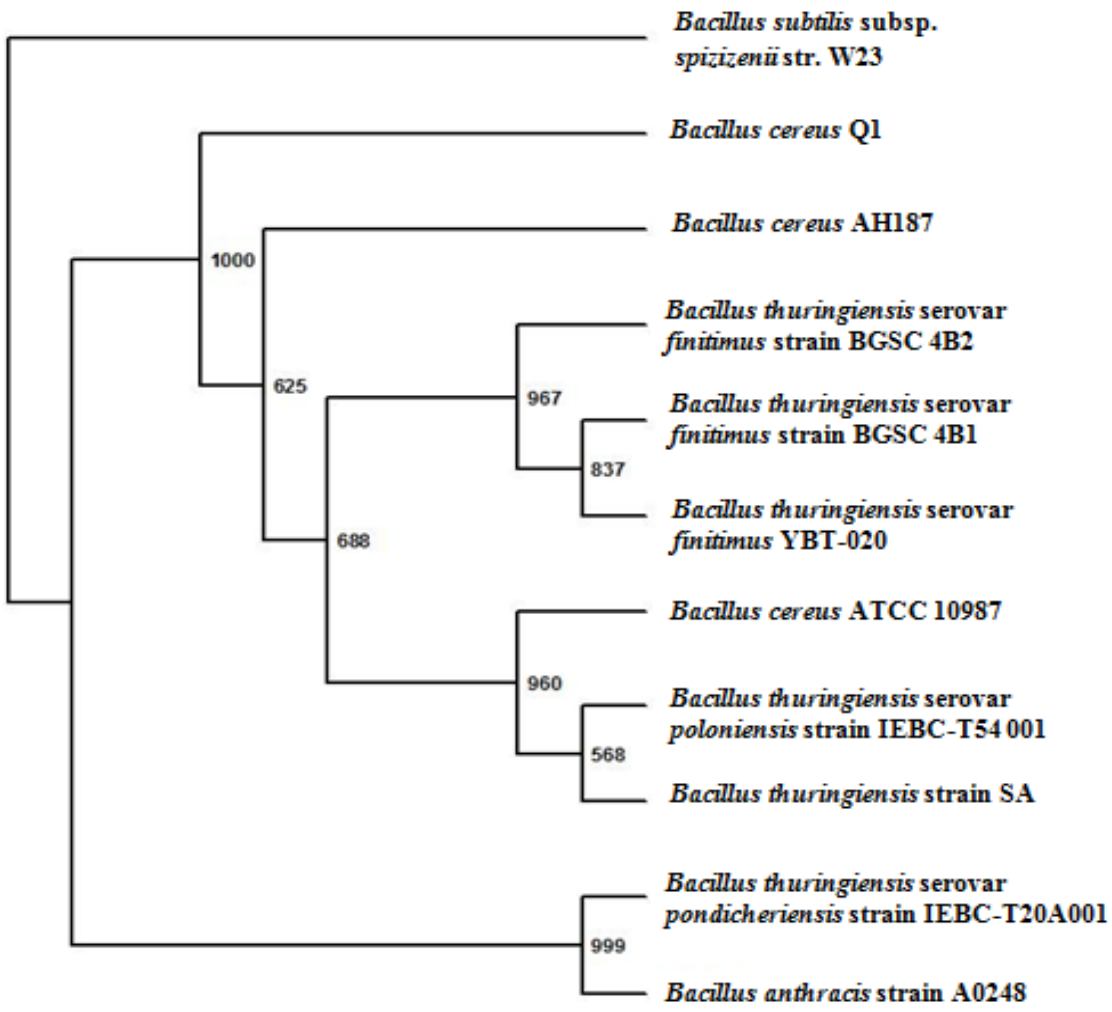


Figure 4.10 Phylogenetic tree of the isolate SA based on the nucleotide sequence of Gyr B gene

4.7 Characterization of Major Protease and Partial Protease Purification

4.7.1 Protease Production

To obtain high amount of crude proteolytic enzyme, bio-reactor was used for this purpose. *B. thuringiensis* SA was cultured overnight in nutrient broth. 10% (v/v) inoculum was transferred into 2% shrimp shells containing 0.1% K_2HPO_4 and 0.05% $MgSO_4.7H_2O$. Then 10% (120 ml) inoculum was poured into 1,200 ml fresh medium (Same culture medium). The condition of bioreactor to produce protease was set at 37^{o}C, 160 rpm and air rate of 2 nl / min. The best period to produce the highest amount of extracellular protease activity from *B. thuringiensis* strain SA was 2 days. The maximum activity of 96 units/ ml was determined after incubation for 2 days (Figure 4.11). The cell free supernatant was collected by centrifugation at 10,000 rpm, 4^{o}C for 20 min and used to analyze the effect of temperature and pH on protease activity and used as crude protein for purification in the further experiment.

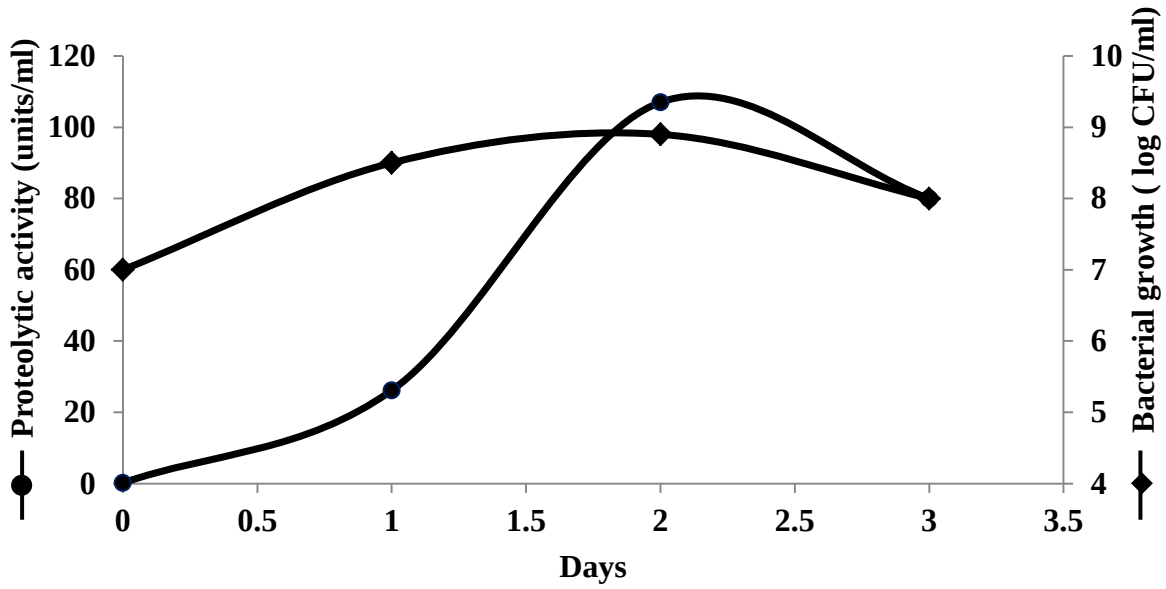


Figure 4.11 Bacterial growth and proteolytic activity of isolate SA cultured in bioreactor at 37^{o}C, 160 rpm and air rate of 2 nl/min for 3 days

To obtain crude protease with high concentration, protein precipitation with ammonium sulfate technique was used for this purpose. The precipitation can remove some contaminated compounds in supernatant and reduce the volume of solution. A soluble protein (Protease) was precipitated completely by addition of 608 g/l ammonium sulfate (80% Ammonium sulfate at 4^{o}C) after the reaction of bioreactor had been stopped. The resultant mixture was kept at 4^{o}C for overnight, and precipitated was collected by centrifugation at 10,000 rpm for 20 min for further experiment. The resultant mixture was dialyzed against distilled water containing 0.02% sodium azide. The resultant mixture was concentrated again on the next day by Lyophilization technique to obtain the powder. The powder was kept at room temperature in desiccator for long time used.

4.7.2 Characterization of Major Protease

4.7.2.1 The Effect of Temperature and pH on Protease Activity

The effects of temperature and pH on the activity of protease are shown in Figure 4.12 and 4.13, respectively. The protease of *B. thuringiensis* strain SA showed activity in a wide range of temperature, from 20 to 70°C (Figure 4.12), but optimum temperature was determined at 50^{o}C. The results revealed that protease had good stability between 4 and 37°C. The studies of the effect of pH on the enzyme activity have also been carried out and the results are shown in Figure 4.13. The protease activity was also detected in a wide range of pHs, from 7 to 11, but the maximum activity was detected at pH of 7.

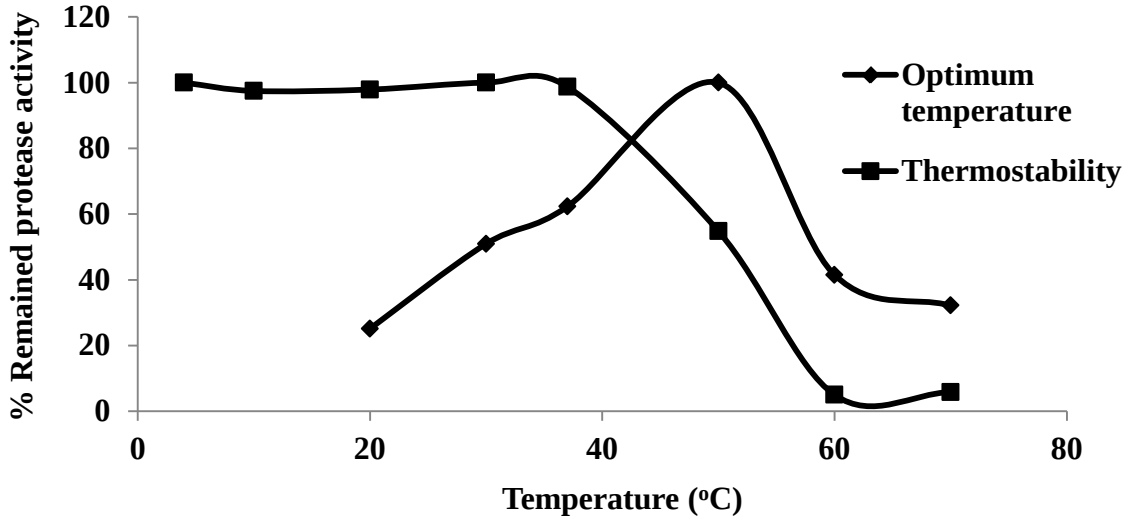


Figure 4.12 Effects of temperature on the protease activity

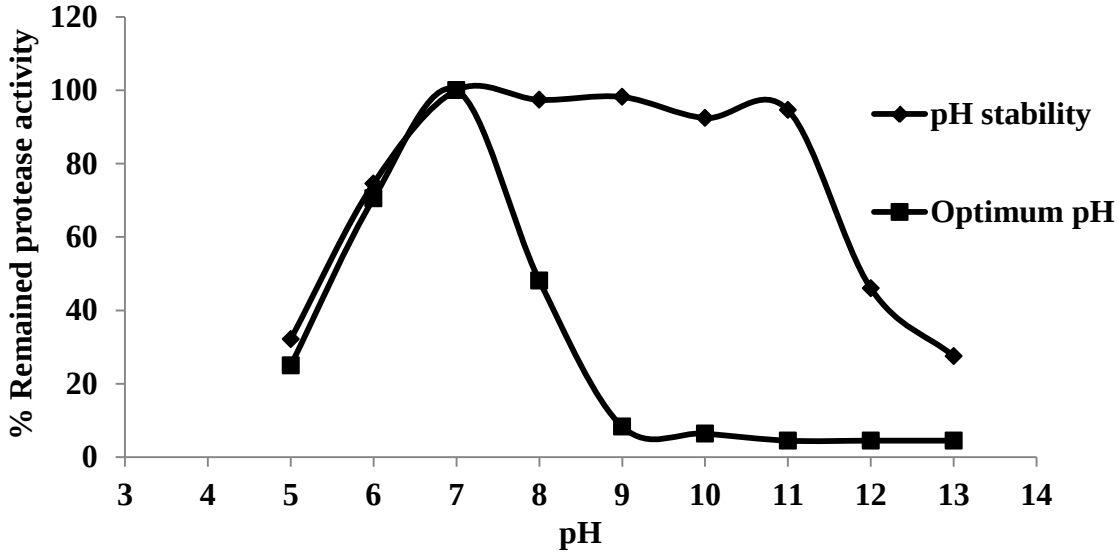


Figure 4.13 Effects of pH on the protease activity

4.7.2.2 Determination of Protease Type

In order to determine type of protease produced by the selected strain, the enzyme activities were also measured in the presence of different concentrations of protease inhibitors and activator. It was found that EDTA only has a significant effect on activity, whereas 2-mercaptoethanol and PMSF did not show significant inhibitory effects against the protease (Table 4.10). Therefore, the major protease produced by the selected strain was a neutral metalloprotease.

Table 4.10 Effect of some reagents on protease activity of the extracellular protease produced by the isolate SA

Chemical	**Function**	**% Remained protease activity at different concentrations of protease inhibitor or activators**		
		5 mM	10 mM	15 mM
Phenylmethylsulfonyl fluoride (PMSF)	Serine protease inhibitor	95.44	96.36	96.15
Ethylenediaminetetraacetic acid (EDTA)	Metalloprotease inhibitor	1.77	1.82	1.21
β-Mercaptoethanol	Activator	95.44	96.36	96.15

4.7.3 Partial Protease Purification

The purification of the protease from the culture supernatant (400 mL) is shown in Table 4.11. The purification steps were combined to give a protease overall purification and overall activity yield of about 1.24 fold and 0.69 %, respectively. The final amount of obtained protease was 0.8 mg. After ammonium sulfate precipitation, obtained protease solution was chromatographed on QAE Toyopearl column, one protein peak containing the protease was washed from column (Figure 4.14). The unpurified protein was dialyzed against distilled water containing 0.02 % sodium azide to remove sodium chloride. The total protein, total activity and purification fold after chromatographed on QAE Toyopearl column were 8.4 mg, 5,604.20 units, and 1.15, respectively. Unpurified solution having protease activity was then passed though Toyopearl HW 50 column (Size exclusion chromatography) (Figure 4.15). After passed this step, protease lost many activities and protein concentration was low. Protease was purified again on hydroxyapatite column and fractioned from column with gradient phosphate solution (0.01-0.8 M PO_4^{2-}) (Figure 4.16). At the final stage of purification, the recovery of the protease activity was rather low. This may be due to the removal of some fractions containing protease with lower specific activity during column chromatography. It is also possible that the protease was unstable on a series of purification.

Table 4.11 Purification of bacterial protease from *Bacillus thuringiensis* SA

Steps	**Total protein (mg)**	**Total activity (Unit)**	**Specific activity (Unit/mg)**	**Recovery (%)**	**Purification fold**
Culture supernatant	148	85,804	579.75	100	1
$(NH_4)_2$ SO_4 ppt	14.13	8,503.02	601.78	9.4	1.03
QAE-Toyopeal	8.4	5,604.20	667.16	5.79	1.15
Toyopearl HW 50	1.1	850	772.72	1.05	1.33
Hydroxyapatite	0.8	600	750	0.76	1.24

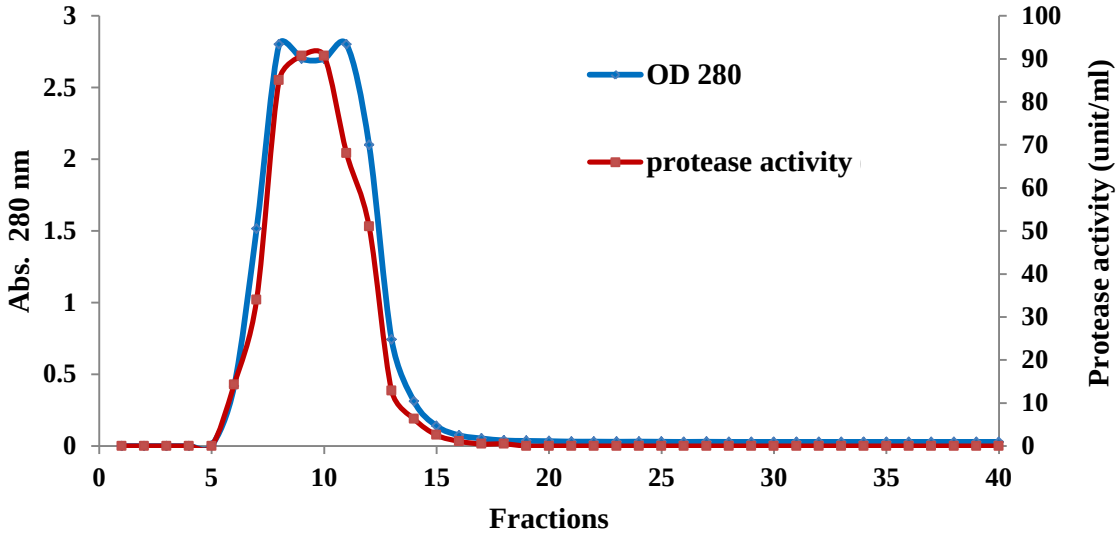


Figure 4.14 Elution profile of protease on QAE TOYOPEAL column

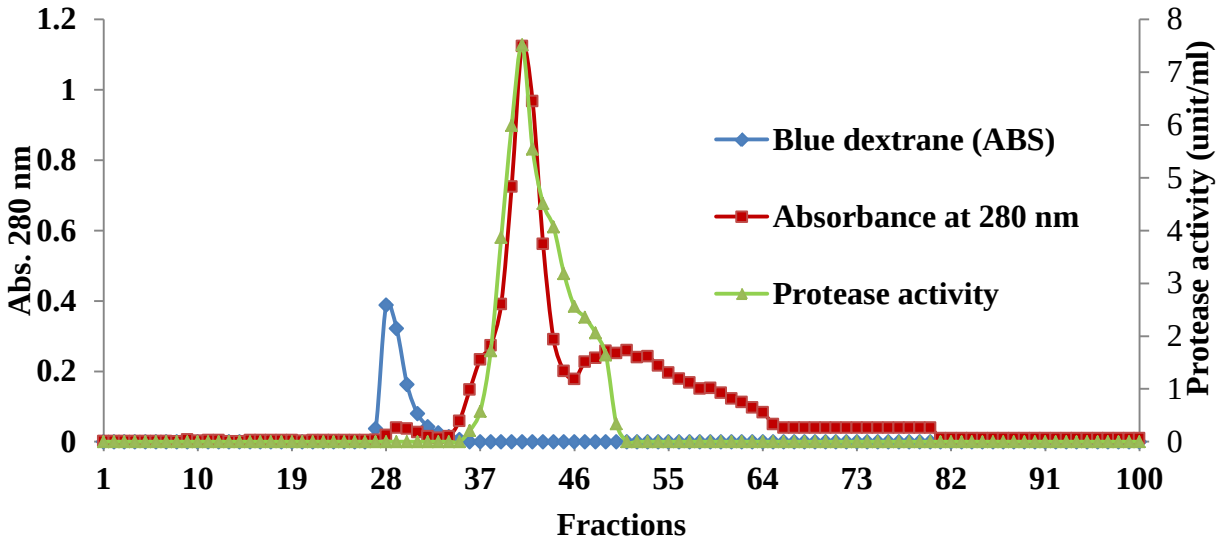


Figure 4.15 Elution profile of protease on Toyopearl HW 50 column

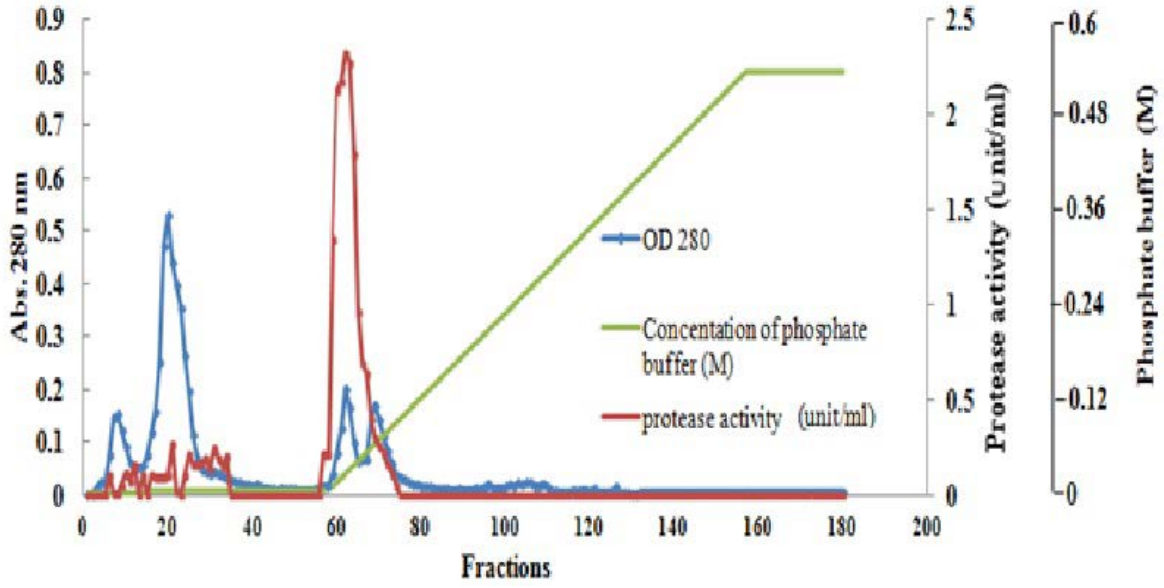


Figure 4.16 Elution profile of protease on hydroxyapatite column

Analysis of the purified enzyme by 10% SDS-PAGE revealed a single band with a molecular mass of about 35 kDa was determined (Figure 4.17). Several published papers have reported for about molecular mass of proteases from other *Bacillus* species; for example, 30.9 kDa thermophilic *Bacillus* strain HS08, 38.0 kDa *Bacillus cereus* KCTC 3674 (Kim et al., 2001), 15.0 kDa *Bacillus subtilis* PE-11 (Adinarayana et al., 2003), 66.2 kDa, 31.0 kDa and 20.1 kDa *Bacillus licheniformis* strains BLP1, BLP2 and BLP3, respectively (Cheng et al., 2006). Those extracellular proteases from *Bacillus* species are different in size depending on protease production medium, cell state and other. In this study, extracellular protease produced by *B. thuringiensis* SA during deproteination of shrimp shells had not only one protease. However, 35 kDa protease has never reported to be the effective protease for degradation of protein in shrimp shells.

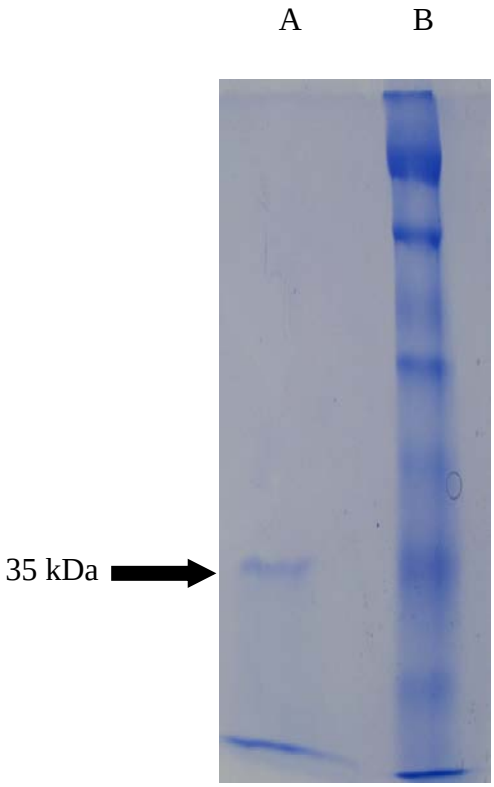


Figure 4.17 SDS-Polyacrylamide gel electrophoresis of protease from *B. thuringiensi* SA, where, lane A: purified protease from *B. thuringiensis* strain SA and lane B: protein markers

4.8 Sources of Acid Producing Bacteria

In the chemical chitin production, decalcification is found that it is the critical step to remove calcium carbonate; major mineral in shrimp shells. The use of HCl to eliminate calcium carbonate is commercially used. Calcium chloride ($CaCl_2$) is obtained after extraction which it is insoluble in water. And the waste water from such process causes environmental problems by producing high acidic waste. To overcome the drawback of traditional decalcification of shrimp shells, shrimp shell fermentation was investigated in this study. Shrimp shell fermentation with acid producing bacteria is a good choice for decalcification and recovery of calcium in the shrimp shells. In this study, lactic acid bacteria were focused as the effective bacteria to ferment and remove calcium from shrimp shells. These bacteria, usually found in decomposing plants and lactic products, produce lactic acid as the major metabolic end-product of carbohydrate fermentation. Acidification can inhibit the growth of spoilage microorganisms. By product from the fermentation is calcium lactate, the action of lactic acid on calcium carbonate. Calcium lactate has various applications in many fields such as medicine and foods. In medicine, calcium lactate is most commonly used as an antacid and also to treat calcium deficiencies. Calcium lactate is added to sugar-free foods to prevent tooth decay. When added to chewing gum containing xylitol, it increases the remineralization of tooth enamel. It is also added to fresh-cut fruits such as cantaloupes to keep them firm and extend their shelf life, without the bitter taste caused by calcium chloride. To obtain lactic acid bacteria, fermented foods in Thailand were found that they are a good source of lactic acid bacteria. Thai traditional fermented sausages; Nham purchased from supermarkets in Bangkok, Thailand were used to screen and isolate lactic acid bacteria. There were 8 Nham samples used for screening of lactic acid bacteria. The properties of Nham samples are shown in the Table 4.12.

Table 4.12 Nham sample characteristics for isolation of lactic acid bacteria

Samples No.	Type of samples	Sources	Important ingredients	pH
1	Nham	Purchased from supermarkets in Bangkok	Minced pork, Pork skin, Chili padi, Garlic, Salt, Rice, and Seasoning powder	~ 4.45
2	Nham	Purchased from supermarkets in Bangkok	Minced pork, Pork skin, Chili padi, Garlic, Salt, Rice, Seasoning powder, and Monosodium glutamate	~ 4.34
3	Nham	Purchased from supermarkets in Bangkok	Minced pork, Pork skin, Chili padi, Garlic, Salt, Rice , Seasoning powder, and Monosodium glutamate	~ 4.35
4	Nham	Purchased from supermarkets in Bangkok	Fish, Cooked rice, Garlic, and Salt	~ 4.51
5	Nham	Purchased from supermarkets in Bangkok	Fish, Cooked rice, Garlic and Salt	~ 4.59
6	Nham	Purchased from supermarkets in Bangkok	Minced pork, Pork skin, Chili padi, Garlic, Salt, Rice, Sugar, Seasoning powder, and Monosodium glutamate	~ 4.36
7	Nham	Purchased from supermarkets in Bangkok	Minced pork, Pork skin, Chili padi, Garlic, Salt, Rice, Seasoning powder, and Monosodium glutamate	~ 4.39
8	Nham	Purchased from supermarkets in Bangkok	Fish, Cooked rice, Garlic, Salt, Seasoning powder, and Monosodium glutamate	~ 4.55

There were 2 major kinds of Nham used in this study; made from fish and pork. pH of Nham samples varied from 4.34 to 4.59. The decreasing of pH in fermented food indicated the complete of fermentation. For Nham, pH is normally at 4.5 due to containing of high protein, it can neutralize pH. Besides reducing pH, acid producing bacteria is found that they are a key to produce a special flavor in fermented foods. Nham samples were transferred into MRS broth to enrich lactic acid bacteria. Pre-primary screening was done to select the lactic acid bacterial colony.

4.9 Screening and Isolation of Acid Producing Bacteria

De Man Rogosa and Sharpe (MRS) medium was used for enrichment of lactic acid bacteria. 1.0 g Nham sample was enriched in 25 ml MRS broth at 37^{o}C for 48 h. Streak plate technique was done on MRS agar containing 0.5% $CaCO_3$. The bacterial colonies surrounded by clear zone were selected and purified. Preliminary identification was done by Gram reaction and catalase test. The bacterial isolates which showed Gram positive and catalase negative were selected and maintained on MRS slant for further experiment. Table 4.13 shows lactic acid bacteria isolated from Nham samples.

Table 4.13 Lactic acid bacteria isolated from Nham samples

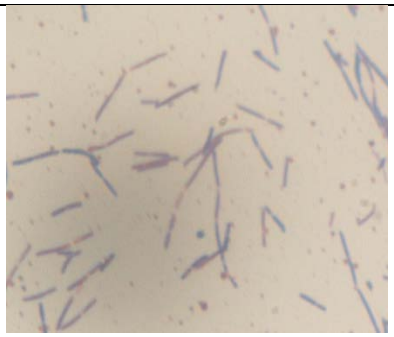
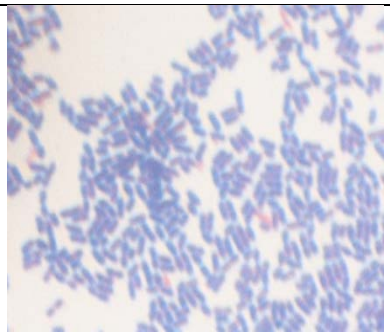
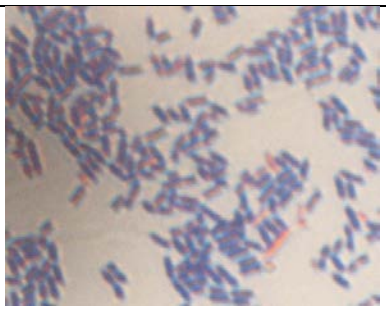
Samples No.	Bacterial isolates	Colony and cell morphologies	Figures
1	L1	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
	L2	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
2	L3	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	

Table 4.13 Lactic acid bacteria isolated from Nham samples (Cont.)

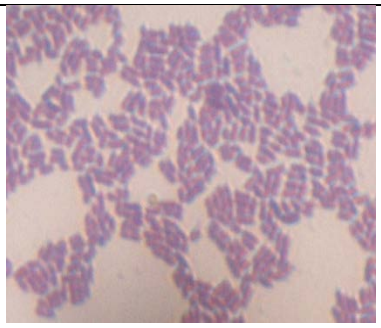
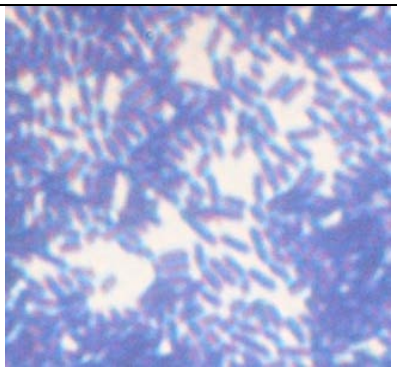
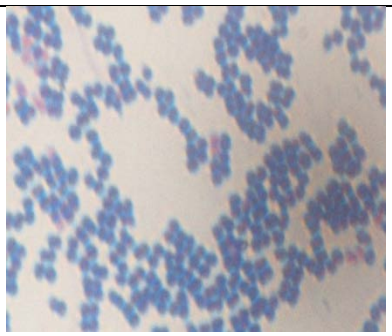
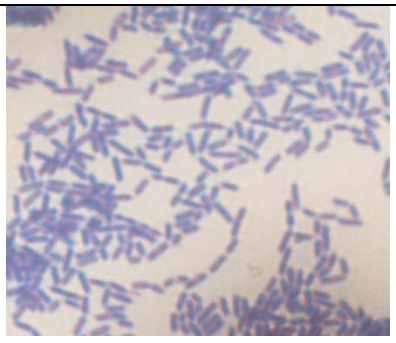
Samples No.	Bacterial isolates	Colony and cell morphologies	Figures
2	L4	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
	L5	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
3	L6	Colony: white, circular, raised Cell: Gram positive, cocci, none spore forming bacteria, catalase negative, no gas production	
	L7	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	

Table 4.13 Lactic acid bacteria isolated from Nham samples (Cont.)

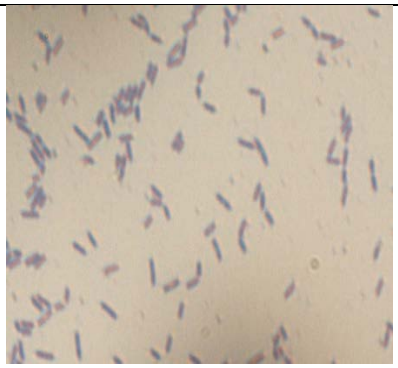
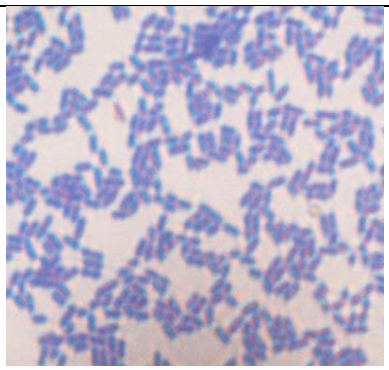
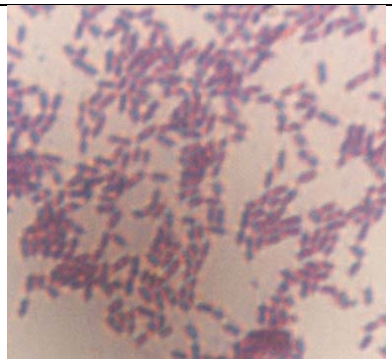
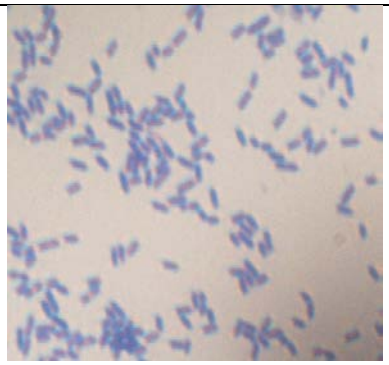
Samples No.	Bacterial isolates	Colony and cell morphologies	Figures
4	L8	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
	L9	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
5	L10	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
	L11	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	

Table 4.13 Lactic acid bacteria isolated from Nham samples (Cont.)

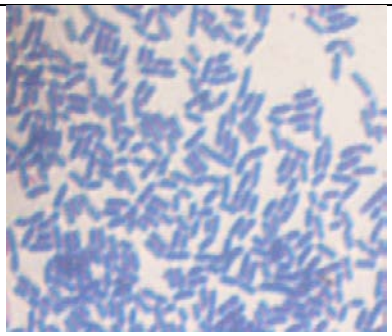
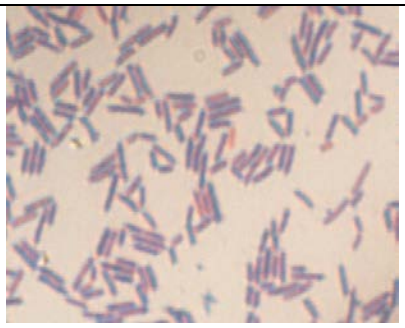
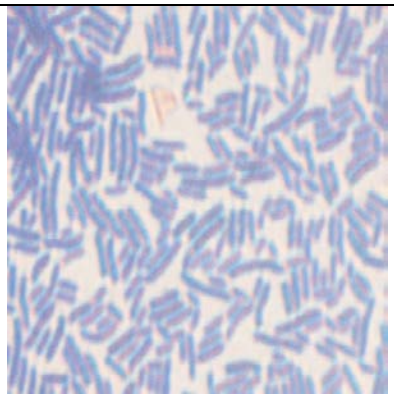
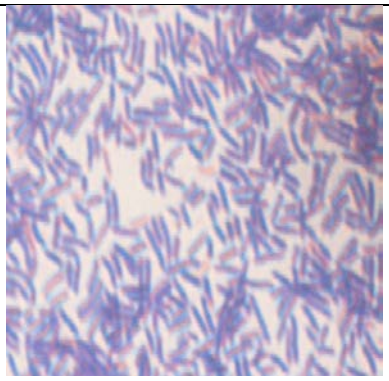
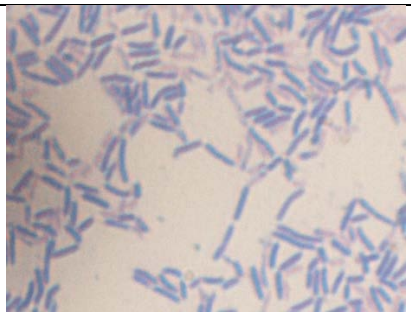
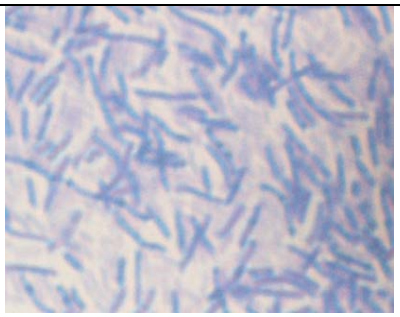
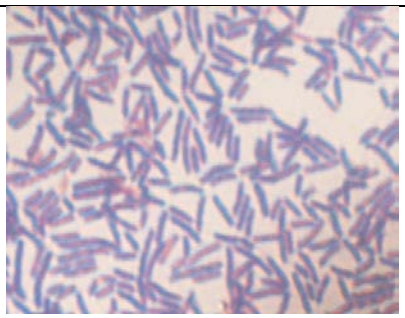
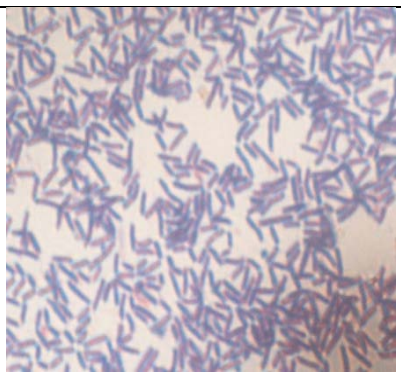
Samples No.	Bacterial isolates	Colony and cell morphologies	Figures
6	L12	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
	L13	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
	L14	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
7	L15	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	

Table 4.13 Lactic acid bacteria isolated from Nham samples (Cont.)

Samples No.	Bacterial isolates	Colony and cell morphologies	Figures
7	L16	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
8	L17	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
	L18	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	
	L19	Colony: white, circular, raised Cell: Gram positive, rod, none spore forming bacteria, catalase negative, no gas production	

19 isolates of acid producing, Gram-positive, catalase-negative and non-spore forming bacteria were isolated from 8 samples of Nham. The lactic acid bacteria (LAB) normally found in Thai fermented meat products are *Lactobacillus* spp., *Pediococcus* spp. and *Micrococcus* spp. which are in the Lactobacillaceae family. The 19 isolates had the common properties of this family.

4.10 Determination of Acid Production

The ability of acid production of the 19 isolates was compared in the MRS broth. All bacterial isolates could not produce gas during cultured in MRS broth; glucose is used as a carbon source. In the fermentation, the bacterial cell culture reached their maximum growth (~10^9 CFU/ml) within one day. The bacterial isolate L1, L4 , L5 , L7 , L8 , L9 , L10, L11, L12, L15, L16, L17, L18, and L19 cloud decrease pH of MRS medium to be lower than pH 3.90; initial pH of MRS broth is 6.60 ± 0.2 (Figure 4.18). However, not only pH is a critical factor to investigate the acid production efficacy. Total tritatable acidity is the important factor to observe acid production ability. After 2 days of fermentation, the isolate L7 gave the highest acid production with TTA value of 2.16% and pH value of the fermenting liquid was 3.68. Therefore, isolate L7 was selected and used for shrimp shell fermentation.

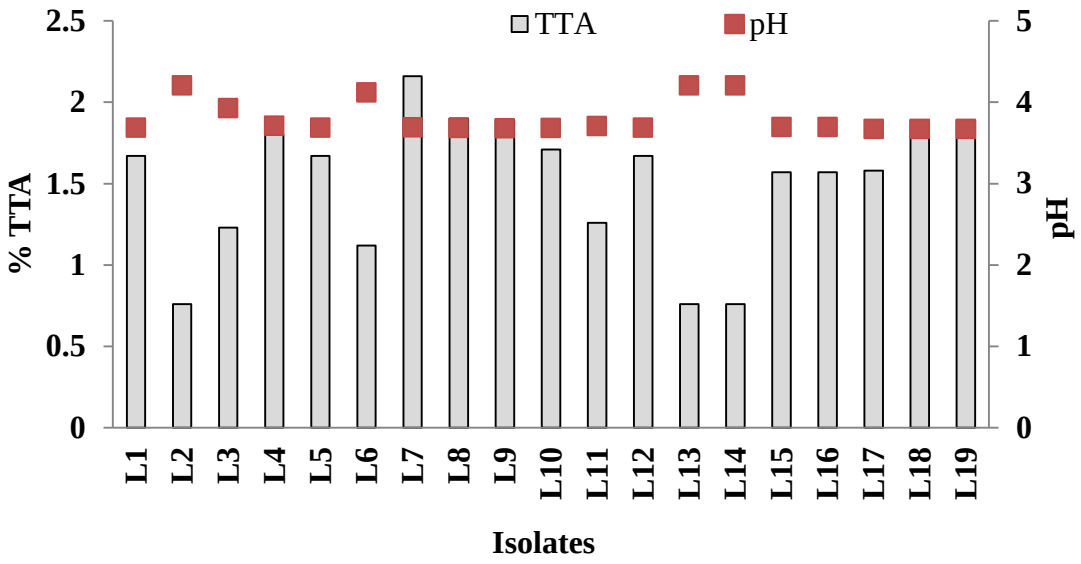


Figure 4.18 pH and TTA (%) of the 48 h MRS broth culture of the 19 acid producing bacterial isolates

4.11 Decalcification of Shrimp Shells

In this study, glucose (Commercial grade) was selected as a carbon source for shrimp shell fermentation. Amount of carbon source (Glucose) in the fermentation plays important roles in growth and lactic acid production by lactic acid bacteria. Table 4.14 shows changing of pH values and total titratable acidity (TTA) during fermentation of shrimp shells for 5 days with different glucose concentrations of 10, 20, 30, 40 and 50% (w/w $_{\text{shrimp shell weight}}$). The results showed that 20% glucose (w/w $_{\text{shrimp shell weight}}$) was determined as an optimum glucose concentration With high glucose concentration (20 and 30% glucose (w/w; $_{\text{shrimp shell weight}}$)), the pH of the fermenting liquid was decreased rapidly form 7.8 to ~4.0 at the second day of fermentation (Table 4.14). After 5 days of fermentation, decalcification and deproteinization efficacies of 97 and 50%, respectively were detected in treatment containing 20, and 30% (w/w $_{\text{shrimp shell weight}}$) (Figure 4.19). The ash content of the products was less than 2% after of fermentation for 5 days (Table 4.15).

Table 4.14 Changing of pH values and total titratable acidity (TTA) during fermentation of shrimp shells for 5 days with different glucose concentrations

Glucose (%)	**pH value**					**(%) TTA**				
	1 Day	2 Day	3 Day	4 Day	5 Day	1 Day	2 Day	3 Day	4 Day	5 Day
10	4.37	4.54	4.58	4.77	5.84	1.95	1.82	2.36	1.95	0.68
20	4.35	4.03	3.89	3.86	3.87	1.75	3.12	3.75	3.85	4.18
30	4.31	4.03	3.89	3.87	3.79	1.89	3.04	4.05	4.19	4.60

pH and TTA of initial shrimp bio-waste were 7.8 and 0.00, respectively.

Table 4.15 Residue ash and crude protein in decalcified shrimp shells after fermentation for 5 days

Glucose (%)	Residue ash (%)	Residue crude protein (%)
10	14.96 ± 0.61	20.01 ± 3.03
20	1.34 ± 0.10	22.94 ± 2.56
30	1.35 ± 0.29	22.80 ± 2.58

Each value is expressed as mean ± s.d. (n = 3)

The excessive amount of glucose did not increase the decalcification efficacy (Figure 4.19), but the remaining glucose after fermentation had affected the production cost due to represent of a high loading BOD waste. Therefore, 20% glucose was considered to be appropriate of glucose concentration for decalcification of shrimp shells.

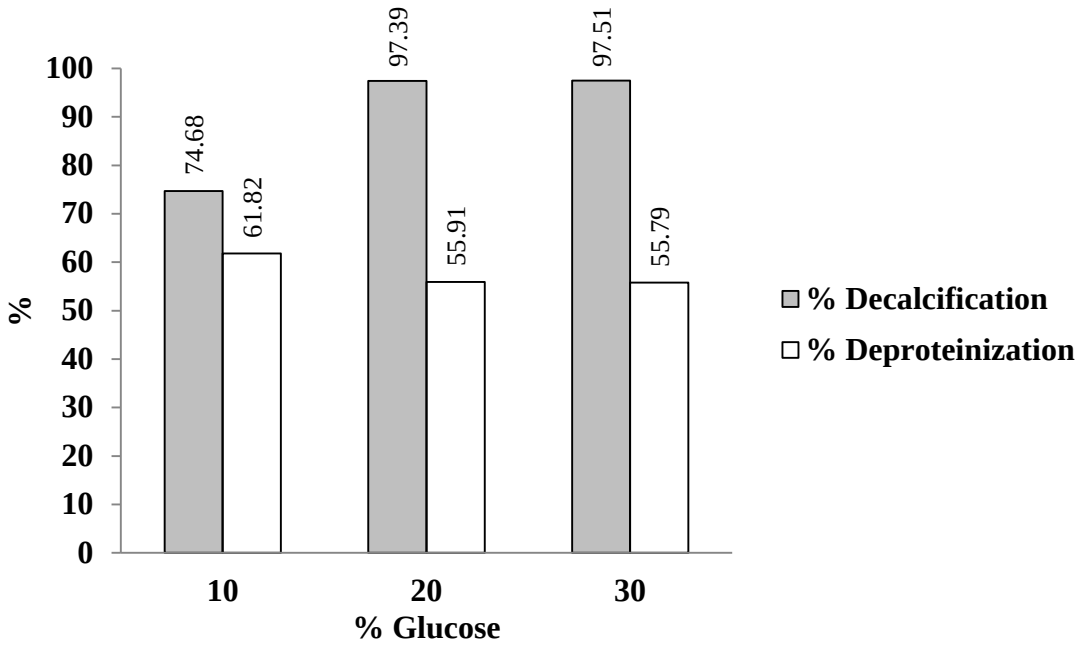


Figure 4.19 Deproteinization and decalcification efficacies of shrimp shells after fermentation by the bacterial isolate L7 for 5 days at 37^{o}C

In addition, inoculum size is considered to be an important factor in the lactic acid fermentation. The effect of inoculum sizes on decalcification of shrimp shells was also

investigated in this study. For increasing of inoculums size, 30% inoculums could decrease the pH value gradually from 7.8 to 4.06 in one day of fermentation (Table 4.16), which indicated that the period requirement for decalcification of shrimp shells could be decreased by increasing of inoculum size.

Table 4.16 Changing of pH values and total titratable acidity (TTA) during fermentation of shrimp shells for 5 days with several sizes of culture inoculum by isolate L7

Inoculums (%)	**pH value**					**(%) TTA**				
	1 Day	2 Day	3 Day	4 Day	5 Day	1 Day	2 Day	3 Day	4 Day	5 Day
10	4.33	3.95	3.92	3.84	3.88	1.41	3.09	3.83	3.24	3.71
20	4.17	3.78	3.78	3.70	3.84	2.16	4.50	4.83	4.05	3.84
30	4.06	3.73	3.72	3.68	3.70	3.69	4.56	4.86	4.59	4.05

pH and TTA of initial shrimp bio-waste were 7.8 and 0.00, respectively.

The treatment with 30% bacterial inoculum had higher decreasing rate of reducing sugar and higher acid production than the other treatments (Table 4.14). However, after one day of fermentation, the demineralization efficacy of the treatment with 30% bacterial inoculum (96.34%) was not significantly different from the treatment with 10% inoculum (96.25%) (Figure 4.20). The ash content of the products was less than 2% after two days of fermentation (Table 4.15). These results lead to an educated assumption that the process of decalcification of shrimp shells by the bioprocess can be finished within two days of fermentation by using effective acid producing bacterial inoculum at 30% and accelerating the fermentation by adding glucose at 20% concentration.

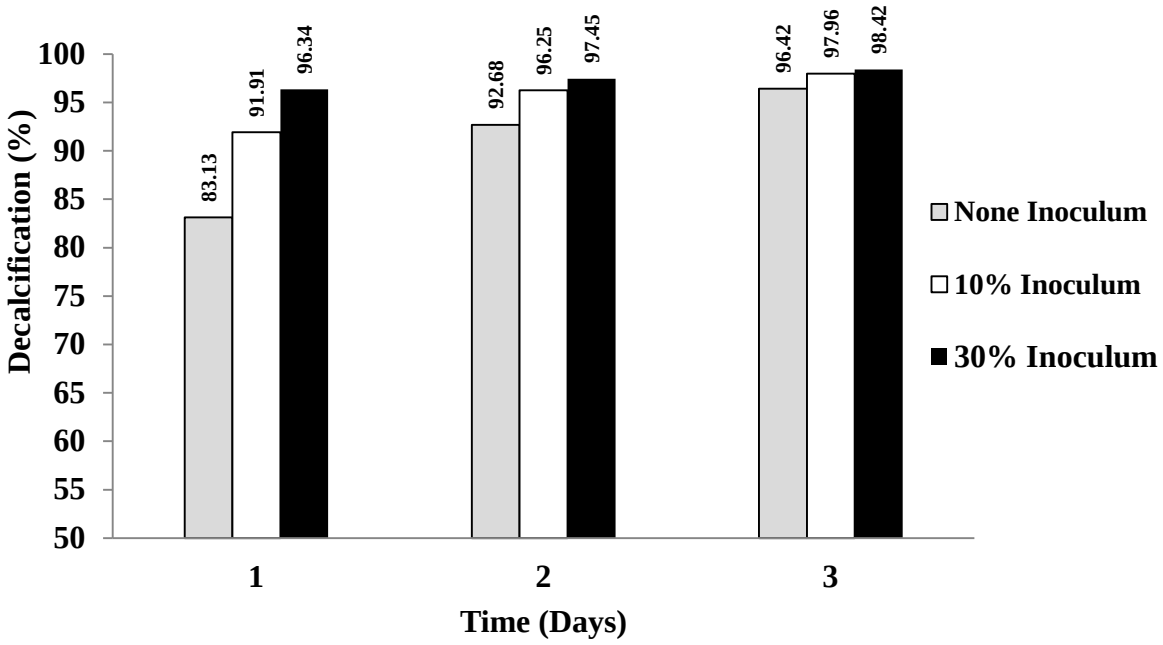


Figure 4.20 Efficacy of shrimp shell decalcification by different acid producing bacterial inoculum sizes

Figure 4.21 Decalcified shrimp shells with lactic acid fermentation for 2 days with 30% inoculums and 20% glucose (w/w $_{\text{shrimp shell weight}}$)

There were many by -products which could be recovered from the fermentation of shrimp shells such as calcium lactate, astaxanthin, and soluble protein. The soluble protein in liquid fermentation could be used for culturing *Saccharomyces cerevisiae* (Appendix E)

4.12The Effect of Carbon Sources on Decalcification of Shrimp Shells

In this section, the effects of different carbon sources on decalcification of shrimp shells were investigated. Beside glucose, sucrose, molasses, and whey were used as carbon sources for shrimp shell fermentation. Sucrose is a disaccharide composed of the monosaccharides glucose and fructose with the molecular formula $C_{12}H_{22}O_{11}$. Various microorganisms can use sucrose as energy for growth. In this study, 10, 15, 20 and 30% sucrose (w/w $_{\text{shrimp shell weight}}$) were applied and used to ferment shrimp shells. The result revealed that 20% sucrose was a suitable carbon concentration for fermentation of shrimp shells. The pH of fermenting liquid was lower than 4.00 and TTA reached 3.5 % after 3 days fermentation in the treatment of 20% and 30% sucrose (Table 4.17). The demineralization efficacy was up to 95% when 20% and 30% sucrose were applied (Figure 4.22). Figure 4.25 shows decalcified shrimp shell by fermentation with isolate L7 using 20% sucrose as carbon source.

Table 4.17 Changing of pH values and total titratable acidity (TTA) during fermentation of shrimp shells for 3 days with different sucrose concentrations

Sucrose	**pH value**			**(%) TTA**		
(%)	1 Day	2 Day	3Day	1 Day	2Day	3Day
10	4.32	4.54	4.53	0.94	1.89	1.89
15	4.3	4.02	4.09	1.48	3.04	3.24
20	4.29	4.09	3.82	1.62	3.04	3.58
30	4.32	3.91	3.84	1.75	3.13	3.59

pH and TTA of initial shrimp bio-waste were 7.8 and 0.00, respectively.

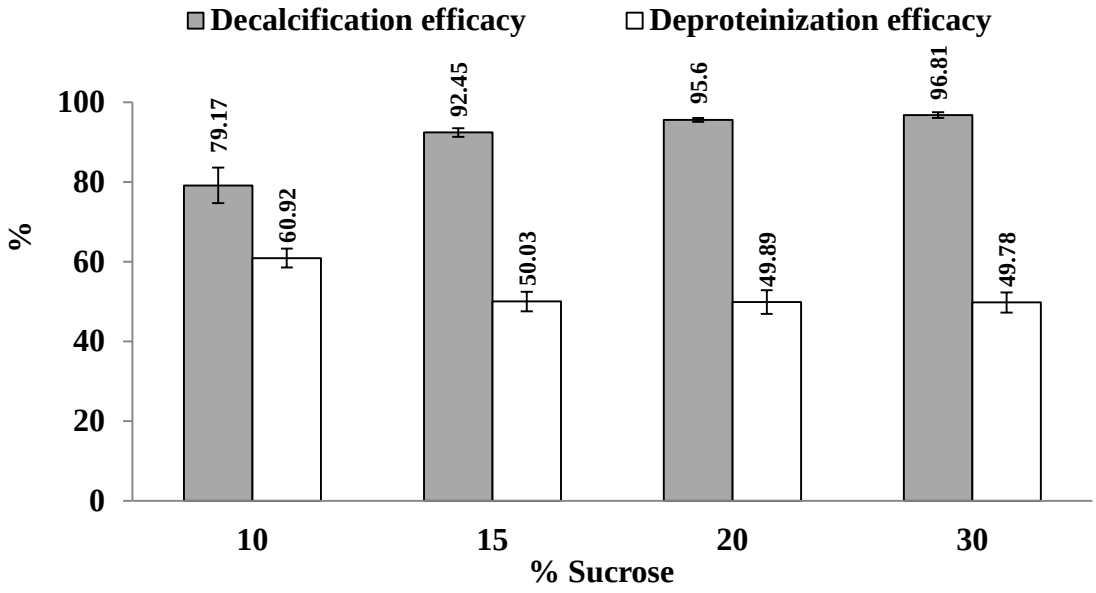


Figure 4.22 Deproteinization and decalcification efficacies of shrimp shells after fermentation by the bacterial isolate L7 for 3 days at 37^{o}C using sucrose as carbon source

Molasses is a viscous by-product of the refining of sugarcane, grapes, or sugar beets into sugar. The quality of molasses depends on the maturity of the source plant, the amount of sugar extracted, and the method employed. For this study, 10, 15, 20, and 30% molasses (w/w $_{\text{shrimp shell weight}}$) were applied and used to ferment shrimp shells. The result revealed that pH of solution could be less than 3.92 when 30 % molasses was supplemented. The pH of fermenting liquid was lower than 4.00 and TTA reached 2.9% after 3 days fermentation in the treatment of 30% molasses (Table 4.18). The decalcification efficacy up to 86% was obtained when 30% molasses were applied (Figure 4.25). However, the use of molasses to ferment shrimp shells had the effect on color properties of the end product. After fermentation, dark decalcified shrimp shells were obtained (Figure 4.26).

Table 4.18 Changing of pH values and total titratable acidity (TTA) during fermentation of shrimp shells for 3 days with different molasses concentrations

Molasses (%)	pH Value			(%) TTA		
	1 Day	2 Day	3 Day	1 Day	2 Day	3 Day
10	4.45	4.91	5.23	0.87	0.6	0.81
15	4.39	4.89	5.51	0.87	0.67	0.87
20	4.39	4.93	5.52	0.81	0.87	1.28
30	4.32	3.91	3.94	1.63	2.36	2.9

pH and TTA of initial shrimp bio-waste were 7.8 and 0.00, respectively.

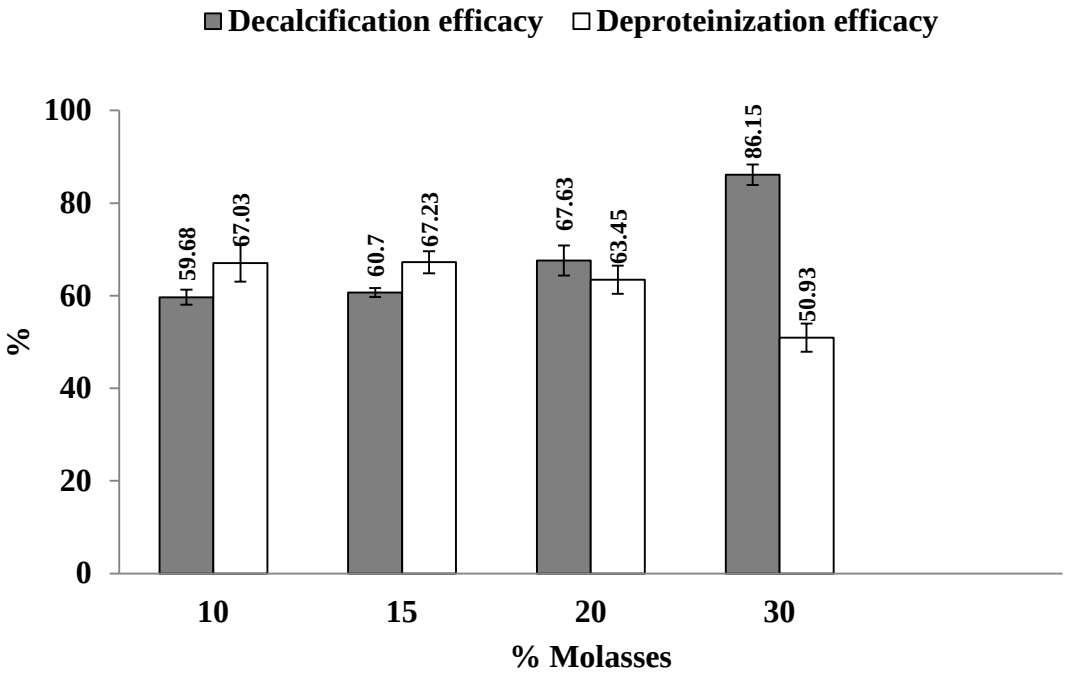


Figure 4.23 Deproteinization and decalcification efficacies of shrimp shells after fermentation by the bacterial isolate L7 for 3 days at 37^{o}C using molasses as carbon source

Whey or milk serum is the liquid remaining after milk has been curdled and strained. It is a by-product of the manufacture of cheese or casein and has several commercial uses. Whey also contains sugar, lactose is a disaccharide sugar derived from galactose and glucose that is found in milk. Lactose makes up around 2~8% of milk (By weight). The whey used in this study had reducing sugar of about 52.90 g/l. The result showed that the use of 100% whey without dilution could demineralize shrimp shells. The pH of fermenting liquid decreased to 3.93 and the demineralization efficacy up to 97% was determined in the treatment using 100% whey (Table 4.19). Figure 4.27 shows decalcified shrimp shells by fermentation with isolate L7 using whey as carbon source.

Table 4.19 Changing of pH values and total titratable acidity (TTA) during fermentation of shrimp shells for 3 days with different whey concentrations

Whey	**pH value**			**(%) TTA**		
(%)	1 Day	2 Day	3 Day	1 Day	2 Day	3 Day
50	4.34	4.31	4.44	1.21	1.35	1.28
75	4.32	4.39	4.28	2.16	2.56	2.9
100	4.34	3.97	3.93	2.29	4.52	5.02

pH and TTA of initial shrimp bio-waste were 7.8 and 0.00, respectively.

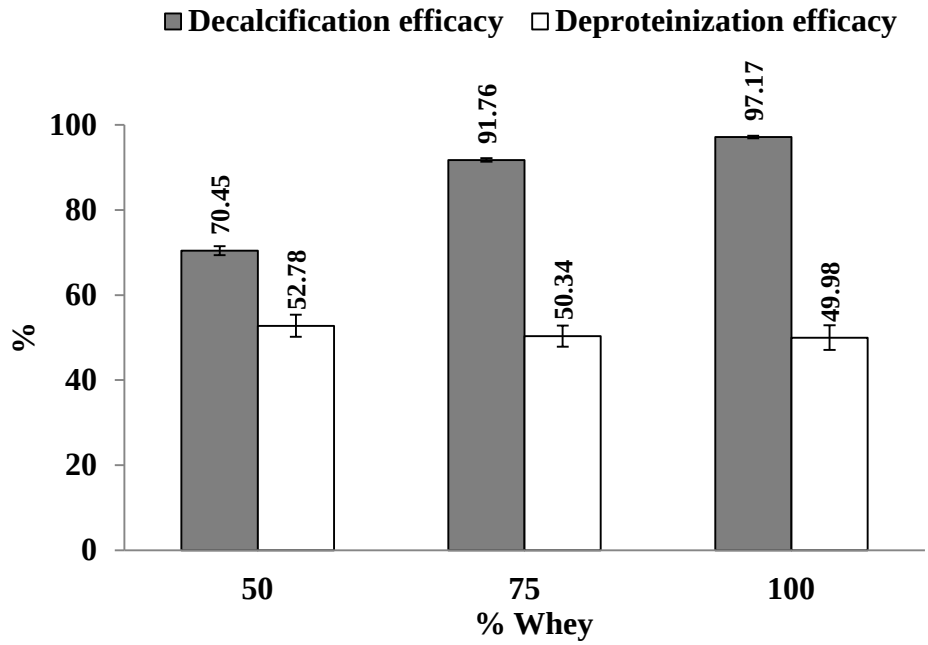


Figure 4.24 Deproteinization and decalcification efficacies of shrimp shells after fermentation by the bacterial isolate L7 for 3days at 37^{o}C using whey as carbon source

Figure 4.25 Decalcified shrimp shells by fermentation with isolate L7 using a sucrose as carbon source

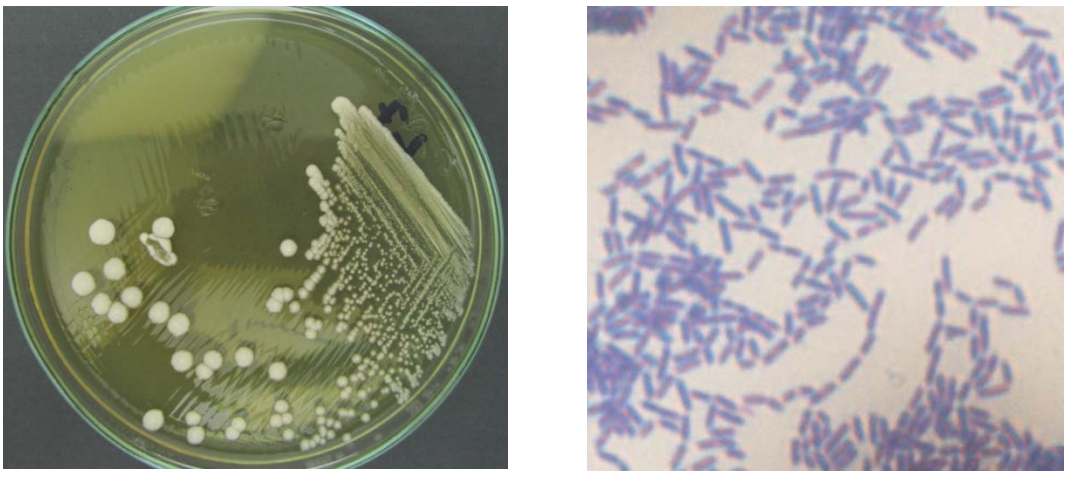
Figure 4.26 Decalcified shrimp shells by fermentation with isolate L7 using molasses as carbon source

Figure 4.27 Decalcified shrimp shells by fermentation with isolate L7 using whey as carbon source

In summary, isolate L7 could use sucrose, lactose (Whey) and molasses to ferment shrimp shells. In this point, fermentation of shrimp shells by isolate L7 might be applied for large scale fermentation which glucose could be replaced with cheap carbon source for saving the production cost.

4.13 Identification of the Selected Acid Producing Bacterial Isolate

Cell morphology and biochemical properties were used to identify the selected bacterial isolate. Biochemical characteristics of isolate L7 are shown in Table 4.20. Isolate L7 showed acid production efficacy in various carbon sources. The 16S rRNA gene of isolate L7 was amplified by PCR method using 800R (18 mers; TAC CAG GGT ATC TAA TCC) and 518F (20 mers; CCA GCA GCC GCG GTA ATA CG) primers. 1570 base pairs of 16S rRNA gene were determined. The BLAST results revealed that isolate L7 was similar to *Lactobacillus pentosus* (Gene blank accession no. NR029133.1) at 99 % homology (Table 4.21). Based on 16S rRNA gene, isolate L7 was identified to be *Lactobacillus pentosus* strain L7. The phylogenetic tree explaining relationship between these isolates and closed species of *Lactobacillus* was constructed by using distance matrix method (Figure 4.30). *L. pentosus* is an industrially important mesophilic LAB that is widespread in the environment and in fermented products of animal and plant origin.



(A) **(B)**

Figure 4.28 The bacterial isolate L7 cultured on MRS agar containing 0.5% $CaCO_3$ after incubation at 37^{o}C for 48 h (A), and Gram stain of the isolate SA (100×)

Table 4.20 Biochemical characteristics of isolate L7

Features	Isolate L7
Colony morphology	Round, creamy, size 0.5 to 0.7 mm
Gram reaction	+
Catalase	-
Cell morphology	Rod shaped
Gas production	-
SIM	-,-,-
Casein utilization	-
Gelatin utilization	-
Growth at	
15°C	+
45°C	+
Growth in	
2% NaCl	+
4% NaCl	+
6% NaCl	+
Acid production	
L-Arabinose	+
D-Cellobiose	+
D-Glalactose	+
D-Gluconate	+
D-Glucose	+
D-Lactose	+
D-Mannitol	+
D-Mannose	+
D-Melebiose	+
L-Rhamnose	+
D-Sorbitol	+
D-Sucrose	+
D-Teharose	+
D-Xylitol	+
D-Xylose	-

Table 4.21 BLAST result of 1525 bases 16S rRNA gene of isolate L7

Accession No.	Strains	Score	% Identity
NR_025447.1	*Lactobacillus paraplantarum* strain DSM	2832	99
NR_029133.1	*Lactobacillus pentosus* strain 124-2	2832	99
NR_042394.1	*Lactobacillus plantarum* strain NRRL B-14768	2826	99
NR_042254.1	*Lactobacillus plantarum* strain DK0 22	2826	99
NR_042676.1	*Lactobacillus fabifermentans* strain LMG *24284*	2823	98
NR_042190.1	*Lactobacillus suebicus* strain CECT 5917	2822	98

CCCGGTTACCGTTAGAGTTTTGATCTGGCTCAGGACGAACGCTGGCGGCGTG
CCTAATACATGCAAGTCGAACGAACTCTGGTATTGATTGGTGCTTGCATCAT
GATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTG
CCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACA
ACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCGGCTATCACTTTTGG
ATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCA
ATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACA
CGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACG
AAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAA
AACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGAC
GGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGG
CGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATC
GGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTA
GCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGT
CTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATT
AGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTGGAGGG
TTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGT

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GACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACATGGAT
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACT
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ACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTT
CGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCG
CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCATGAAAAGTTTGTAACACCCAAAGTCGGTTGGGGTAACCTTTTT
AGGAACCAGCCGCCTAAGGTGGGACAGATGATTAAGGTGAAGTCGTACAG
GGGAATCACCTATAGAGGGGGGAATTATAACCTT

Figure 4.29 DNA sequence of 16S rRNA gene of isolate L7

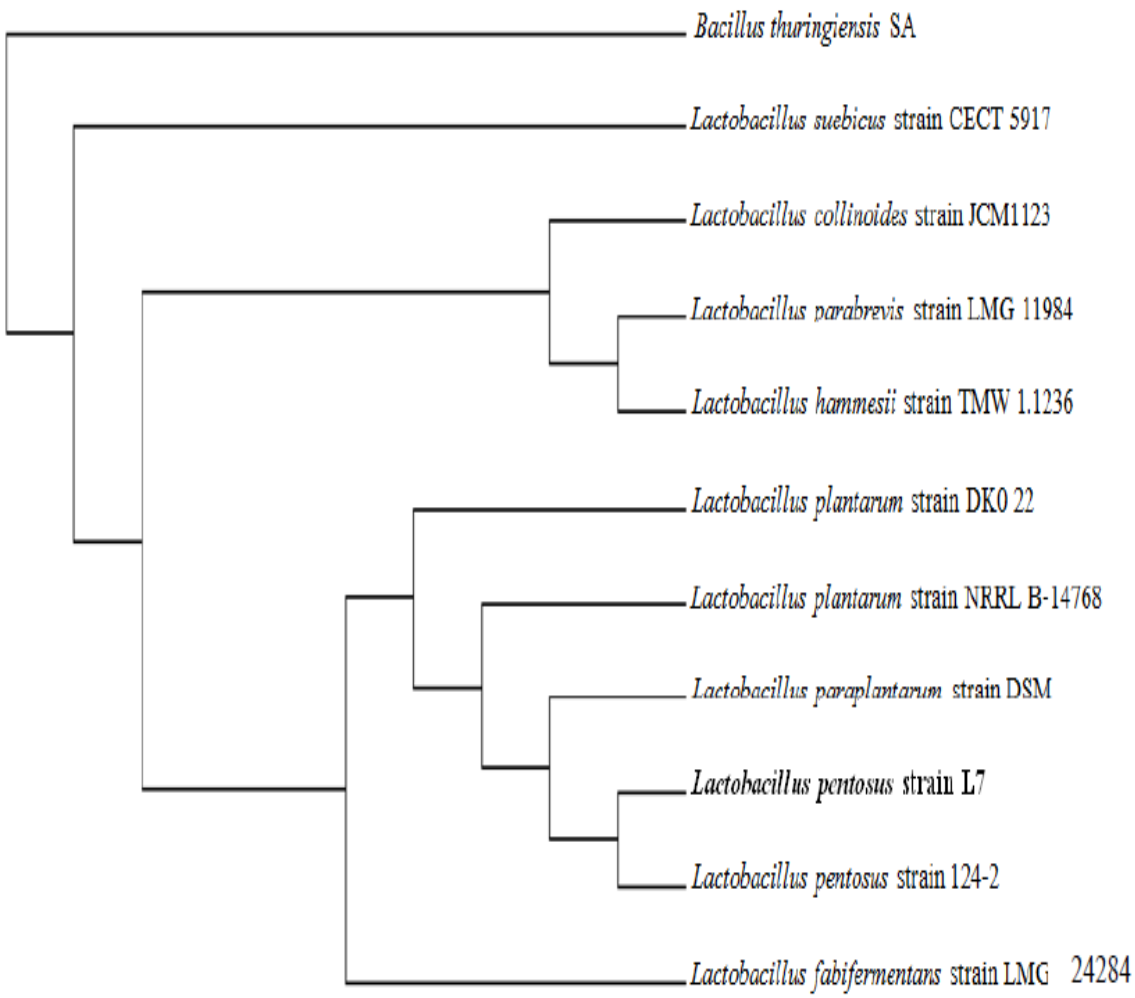


Figure 4.30 Phylogenetic tree of the isolate L7 based on the nucleotide sequence of 16S rRNA gene

4.14 Chitin Purifications

Chitin (β-1,4-linked N- acetyl glucosamine (GlcNAc) is the most abundant natural polysaccharide on earth after cellulose and has wide range applications in different fields such as waste water treatment, material science, microbiology, agriculture, drug delivery and food technology. In the commercial extraction process, chitin is usually isolated by a simple process which involves the use of alkaline and acid to deproteinize and decalcify the shrimp shells, it is considered to be non -environmental friendly process. In this study, chemical processes were used to prepare chitin.

4.14.1 Chemical Chitin Extraction

450 ml of 4% HCl was added to 100 g of shrimp shells and kept at room temperature for 4 h to eliminate inorganic components in the shells. Then, the decalcified shells was separated and washed many times with distilled water. The protein and ash contents of decalcified shrimp shell**s** were determined in the Table 4.22. The result showed that calcium carbonate could be removed completely from the shrimp shells. Ash content of 0.47 ± 0.12% was determined after chemical decalcification; initial ash content in shrimp shells were 27.92 ± 0.94% (Table 4.1). However, crude protein content of decalcified shrimp shells was 30.86 ± 2.78% which it was higher than crude protein content in the raw shrimp shells (25.92 ± 2.03%). This point indicated that HCl extraction had low efficacy to remove protein from shrimp shells. Figure 4.31 shows the decalcified shrimp shells after treating with 4% HCl. Chitin flakes were obtained by deproteinization of decalcified shrimp shells by adding 1,000 ml of 5% NaOH to 100 g of dried decalcified shells. The mixture was shaking in 90^{o}C water bath for 12 h. The chitin flakes were washed with trap water until pH of rinsed water became ~ 7.0. At the end of process, chemically processed chitin had residue crude protein and ash at 0.34 ± 0.10% and 0.57 ± 0.14%, respectively (Table 4.22). Deproteinization and decalcification efficacies of chemically processed chitin were 99.73 ± 0.08 % and 99.02 ± 0.12%, respectively (Table 4.22). Figure 4.32 shows chitin flakes obtained by chemical extraction.

Table 4.22 Chemical properties of decalcified shrimp shells and chitin flakes obtained from chemical extraction

Components (%)	Decalcified shrimp shells	Chitin flakes
Total kjeldahl nitrogen	9.37 ± 0.18	6.35 ± 0.12
Crude protein	30.86 ± 2.78	0.34 ± 0.10
Ash	0.47 ± 0.12	0.57 ± 0.14
Deproteinization efficacy	10.61 ± 0.84	99.73 ± 0.08
Decalcification efficacy	98.94 ± 0.15	99.02 ± 0.12
Remaining Residue	62.53 ± 2.35	20.01 ± 0.45

Each value is expressed as mean ± s.d. (n = 3)

Figure 4.31 Decalcified shrimp shells after treated with 4% HCl

Figure 4.32 Chemically processed chitin flakes

4.14.2 Preparation of Shrimp Head Extract Solution (SHES)

Chemical composition of shrimp heads is shown in Table 4.23. The available protein content in shrimp heads could be used as growth supplement for microorganisms. Various kinds of process were applied for extracting protein from shrimp heads which the use of proteolytic enzyme was a favorite method to recover protein hydrolyzate. However, proteolytic enzyme is expensive and unable to reuse after extraction leading to high production cost. To solve this problem, heat extraction; simple and cheap processes were used to extract protein from shrimp heads. The process to extract macromolecules was explained in the "research methodology". SHES obtained by heat extraction process was cheap and easily to prepare. SHES contained crude protein of 1.03 ± 0.06% (Table 4.24) which it could well support the growth of bacteria. The residue after extraction still had some nutritional values (Table 4.23) which could be used for other applications such as animal feed production.

Table 4.23 Chemical properties of shrimp heads before and after extracted by heating

Components (%)	**Shrimp heads**	**Shrimp head after extracted by heating**
Moisture	86.13 ± 2.19	-
Total kjeldahl nitrogen	9.06 ± 0.12	8.03 ± 0.15
Crude protein	40.74 ± 2.66	37.97 ± 3.67
Lipid	13.87 ± 1.12	5.01 ± 0.28
Ash	22.21 ± 0.61	27.54 ± 1.89

Each value is expressed as mean ± s.d. (n = 3)

Table 4.24 Composition of shrimp head extract solution (SHES)

Properties	Components (%)
Total solid	1.51 ± 0.13
Total kjeldahl nitrogen	0.16 ± 0.01
Crude protein	1.03 ± 0.06
Lipid	0.43 ± 0.12
Ash	0.26 ± 0.05

Each value is expressed as mean ± s.d. (n = 3)

Therefore, SHES was used as a major nitrogen source in culture medium for bacterial innoclum preparation.

4.14.3 Determination of *L. pentosus* L7 Growth in Various Culture Media Having SHES as a Major Component

A 24 h culture of *L. pentosus* L7 (0.1%) in MRS broth was inoculated into: MRS; SHES; SHES plus 2% glucose; SHES plus 2% NaCl; SHES plus 2% glucose and NaCl; and modified MRS medium. The growth curves of *L. pentosus* L7 in these media were performed using the spread plate technique on MRS agar (Figure 4.33). The SHES could not support the growth of *L. pentosus* L7 unless a carbon source such as glucose was supplemented. The modified MRS medium which used SHES to replace peptone, beef extract, and yeast extract could satisfactorily promote the growth of *L. pentosus* L7 similar to the MRS medium. *L. pentosus* L7 cell numbers reached 9.2 ± 0.2 log CFU/ml after 24 h cultivation. SHES plus 2% glucose could also promote the growth of *L. pentosus* L7; cell numbers reached 8.5 ± 0.2 log CFU/ml after 24 h cultivation. SHES plus 2% glucose could save on the cost of inoculum medium; therefore, SHES plus 2% glucose medium was selected as the cultivation medium to prepare the inoculum for lactic acid fermentation of shrimp shells.

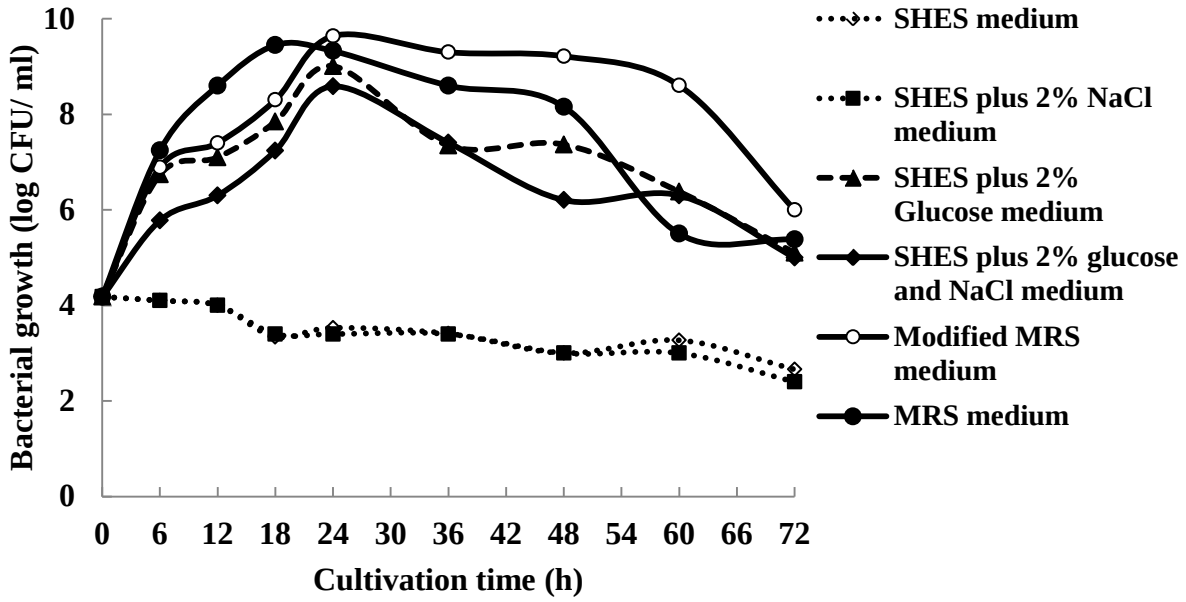


Figure 4.33 Growth of *L. pentosus* L7 in 6 different culture media

4.14.4 Determination of *B. thuringiensis* SA Growth in SHES Compared to Nutrient Broth (NB) and Tryptic Soy Broth (TSB)

When a 24 h culture of 0.1% *B. thuringiensis* SA in NB was inoculated into SHES, NB and TSB, the three media gave similar growth support for the bacteria. The cell numbers of *B. thuringiensis* SA reached 8.6 ± 0.2 log CFU/ml after 24 h cultivation indicating that SHES would be suitable for use as the culture medium for *B. thuringiensis* SA.

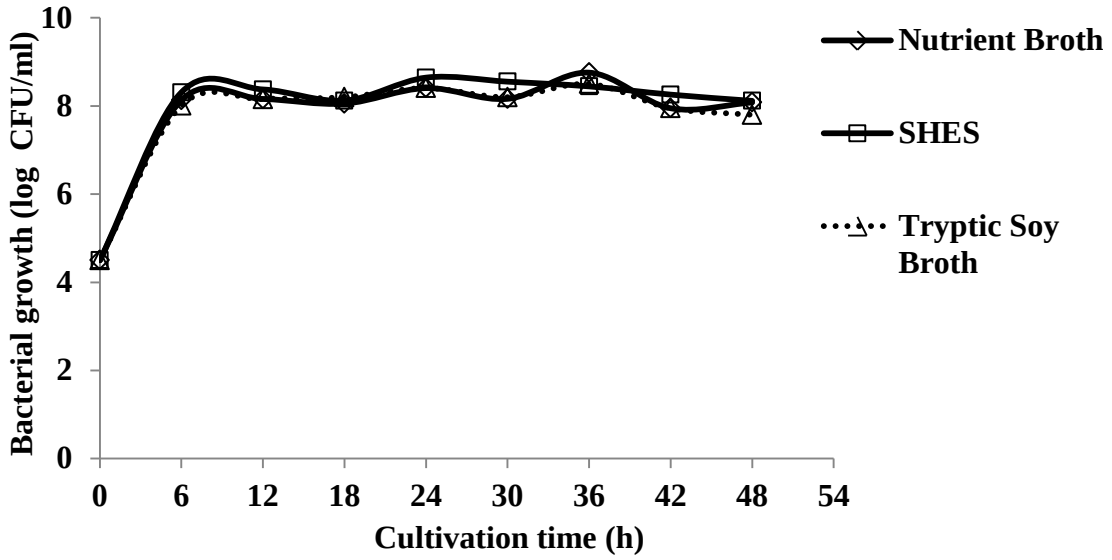


Figure 4.34 Growth of *B. thuringiensis* SA in nutrient broth, shrimp head extract solution (SHES), and Trytic soy broth

4.14.5 Biological Chitin Purification

Removal of protein and calcium in the shells are critical steps in chitin purification of shrimp shells. Lactic acid fermentation was used as the first process in a two-step biological chitin extraction. According to section 4.11, 20% (w/w $_{\text{initial shrimp waste}}$) glucose was determined to be optimum condition to ferment shrimp shell. High inoculum level had the significant effect on the production cost. From the section 4.14.3, *L. pentosus* L7 could grow well in the SHES plus 2% glucose. This medium has SHES and glucose in the formula, therefore, the price of the medium is much cheaper. The lactic acid fermentation of shrimp shell flake was performed by suspending 500 g of shrimp shell flake, 100 g glucose, and 10 g NaCl and accompanying with 500 ml of *L. pentosus* L7 starter. The fermentation was done in a closed container incubating at 37^{o}C for 48 h. The changing in amount of reducing sugar, lactic acid, and pH of the fermenting liquid were determined (Figure 4.35).

The initial pH of 7.00 ± 0.10 fell to 3.90 ± 0.10 after 48 h, and continued to decrease slightly upon further incubation. The acidity was highest after 48 h, and then remained at that level. The reducing sugar in the fermenting liquid remained at only 4.00 ± 0.10 g/L after 72 h. In the first 24 h, the number of *L. pentosus* L7 cells increased to 11.00 ± 0.20 log CFU/ml indicating significant growth. Over the first 24 h, the ash content in shrimp shells sharply decreased from 27.92 ± 0.94% to 3.82 ± 0.35% (Table 4.25). The results suggested that the fermentation process ended after 48 h as indicated by constant pH and lactic acid concentration (Figure 4.35). At the end of fermentation, the decalcified shrimp shells had crude protein and ash contents of 23.24 ± 2.52% and 1.44 ± 0.41%, respectively (Table 4.25). The decalcified shrimp shells were further processed by a deproteinization step via the proteolytic activity of *B. thuringiensis* SA. The proteolytic activity and soluble protein in the extraction liquid were examined (Figure 4.36). The highest proteolytic activity, 290.10 ± 15.80 units/ml was detected after 48 h and the highest amount of soluble protein in the extraction liquid was found after 72 h; this indicated that the deproteinization could be considered finished after 72 h. At the end of the process, decalcification and deproteinization efficiencies of the two-step biological extraction were 98.05 ± 0.31% and 96.80 ± 0.67%, respectively (Figure 4.38).

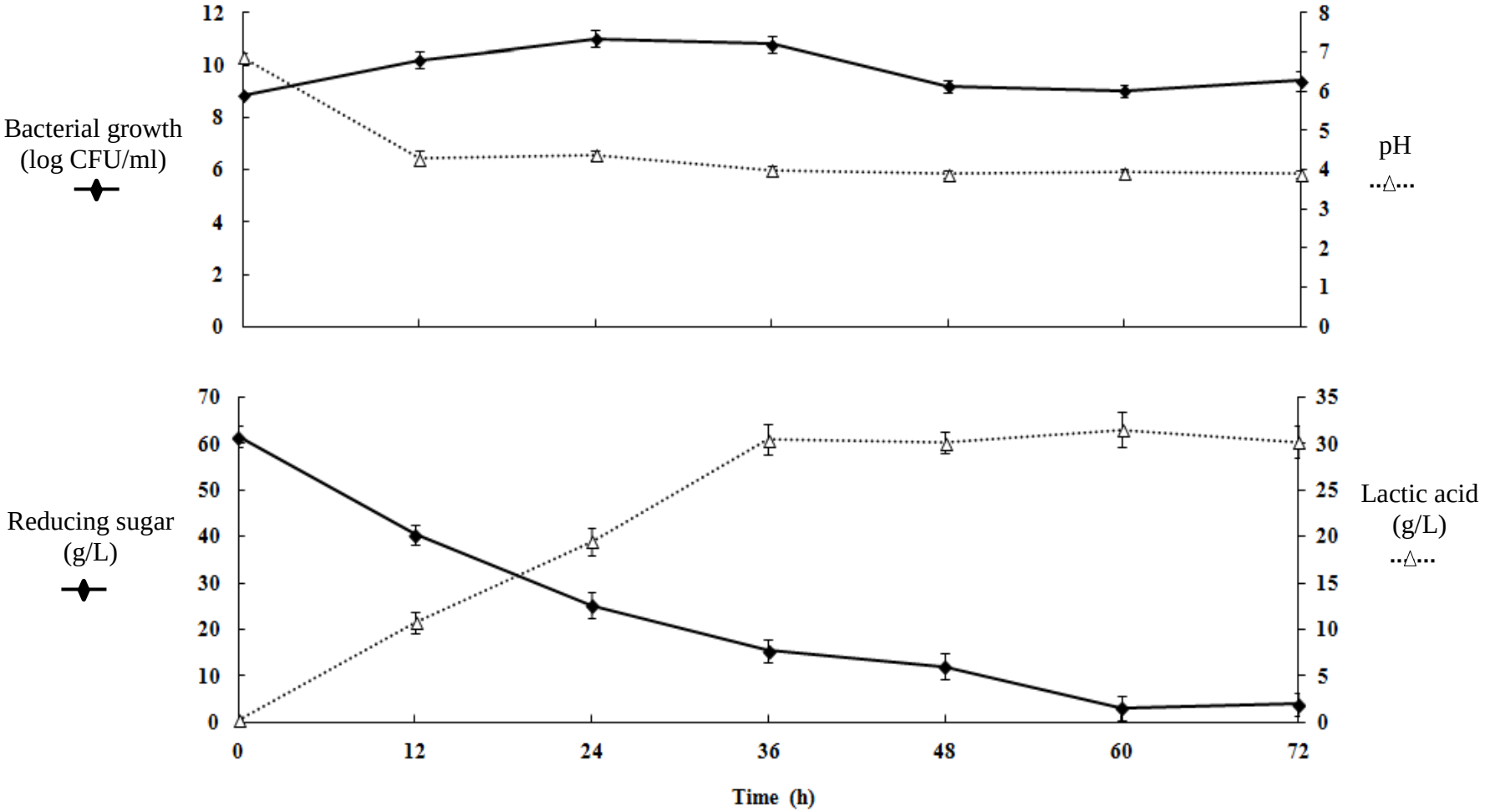
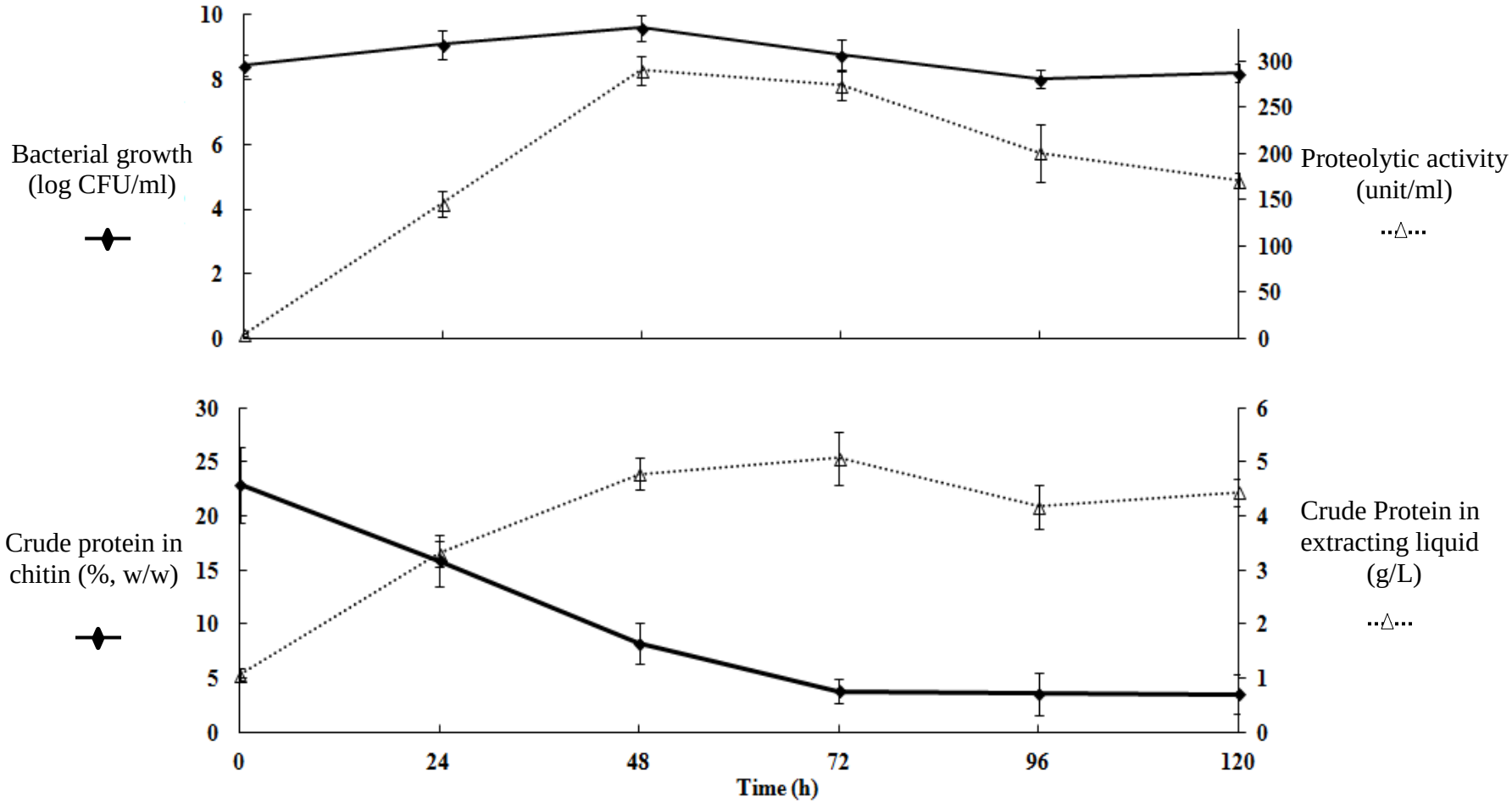


Figure 4.35 Changing in pH, Lactic acid and reducing sugar in the fermented liquid during decalcification of shrimp shells by *L.pentosus* L7



Figigure 4.36 Deproteinization of decalcified shrimp shells by *B. thuringiensis* SA

Table 4.25 Crude protein and ash contents in decalcified shrimp shells and raw chitin during purification by two steps biological process

Biological process	Crude protein (%, w/w)	Ash content (%, w/w)
L. pentosus L7 fermentation		
1 day	26.31 ± 1.24	3.82 ± 0.35
2 days	23.24 ± 2.52	1.44 ± 0.42
Deproteinization with *B. thuringiensis* SA		
1 day	16.41 ± 2.48	1.47 ± 0.41
2 days	7.97 ± 1.24	1.49 ± 0.40
3 days	3.80 ± 1.28	1.72 ± 0.37

Each value is expressed as mean ± s.d. (n = 3)

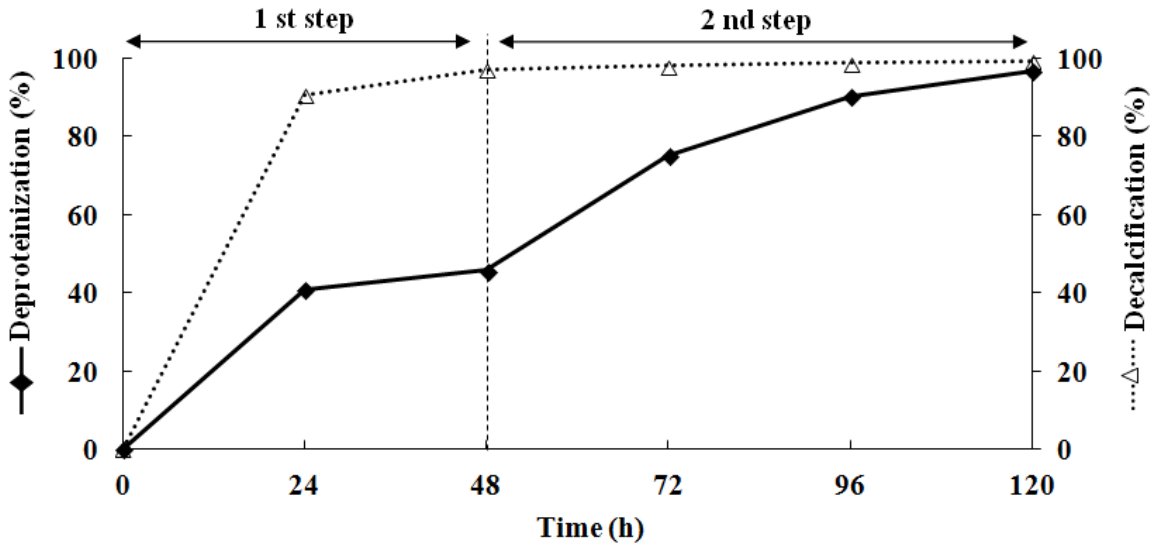


Figure 4.37 Deproteinization and decalcification efficacies of shrimp shells during the 1 st step fermentation with *L. pentosus* L7 and the 2 nd step deproteinization with *B. thuringiensis* SA

Figure 4.38 Biologically purified chitin

Table 4.26 Properties of biologically purified chitin

Properties	Biologically purified chitin
pH	7
Appearance	White and light yellow
TKN (%,w/w)	0.62 ± 0.45
Crude protein (%,w/w)	3.80 ± 1.28
Ash (%,w/w)	1.72 ± 0.37
Deproteinization efficacy (%)	96.80 ± 0.67
Decalcification efficacy (%)	98.05 ± 0.31

Each value is expressed as mean ± s.d. (n = 3)

Table 4.26 shows properties of biologically purified chitin. The chitin flakes made from same lot of shrimp shells using chemical process extraction had residual calcium and

crude protein content at 0.57 ± 0.14% and 0.34 ± 0.10% respectively. The chemical process could diminish the protein content of shrimp shells and calcium of shells better than the biological process.

A fast removal of proteins from shrimp abdominal shells is necessary to avoid spoilage and the development of a bad smell. Shrimp shell fermentation by lactic acid bacteria for decalcification and deproteinization would be an alternative approach for inhibition of spoilage microorganisms. By fermentation of shrimp shells with lactobacilli, a decalcification efficiency of up to 86% has been reported (Adour et al., 2008; Bautista et al., 2001; Bhaskar et al., 2007; Cira et al., 2002; Jung et al., 2007; Rao et al., 2000; Xu et al., 2008). In the present study, decalcification efficacy with *L. pentosus* L7 reached 97% but required 10% (w/w) glucose. This is in accordance with previous reports (Bhaskar et al., 2007; Jung et al., 2007; Xu et al., 2008): for example, 15% (w/w) was the optimum glucose concentration for *Pediococcus acidilactici* fermentation to remove calcium from shrimp shells (Bhaskar et al., 2007), while 2.5g crab shells were decalcified with 50mL of 10% glucose (solid to liquid ratio of 1 : 20) (Jung et al., 2007); and for decalcification of *P. monodon* shells, 0.54 g glucose per g of dried shells was added to decrease pH to 4.6 (Xu et al., 2008). The decrease of residual proteins in shrimp shells after fermentation indicated that deproteinization of the shrimp shells occurred spontaneously, together with decalcification by the proteolytic activity of *L. pentosus* L7 or by the in situ proteases in the bio-waste (Xu et al., 2008). The presence of the lactic acid bacterium *L. pentosus* L7 (9.00 ± 0.20 log CFU/mL) in the protein and calcium rich liquor portion indicates that it could be used as a food supplement for humans, animals, or microorganisms (Aranaz et al., 2009).

In the deproteinization step, *B. thuringiensis* SA produced high protease activity (290.10 ± 15.80 unit/mL) during deproteinization of decalcified shrimp shells. The liquid fraction should be collected and concentrated to obtain the crude protease. *B. thuringiensis* SA was found to produce parasporal crystal proteins during the sporulation stage; the crystal protein might be recovered from the extracting liquid after deproteinization of decalcified shrimp shells.

The production of chitin isolated by a two-step biological purification process for decalcification and deproteinization is not commercially used; but the process has good potential to create biologically purified chitin with a high grade of purity.

The disadvantages of a two-step biological process are overall longer purification time (5 days) and higher costs due to the requirement of sterilize process and a carbon source in deproteinization and decalcification steps. However, the wastes from the biological processing are not harmful to humans or the environment, and useful by-products such as protein hydrolysate, calcium lactate, astaxanthin, crude protease, and parasporal crystal proteins during purification could be obtained from the methods used in the present study; whereas chemical process creates toxic waste and causes some depolymerization of the chitin, which influences its molecular weight and viscosity, leading to lower viscosity chitosan after deacetylation. The two-steps biological process gave a good quality of chitin flakes which could be further processed to be a high quality of chitin powder; therefore, this process should be applied for the industrial scale chitin production in the future.

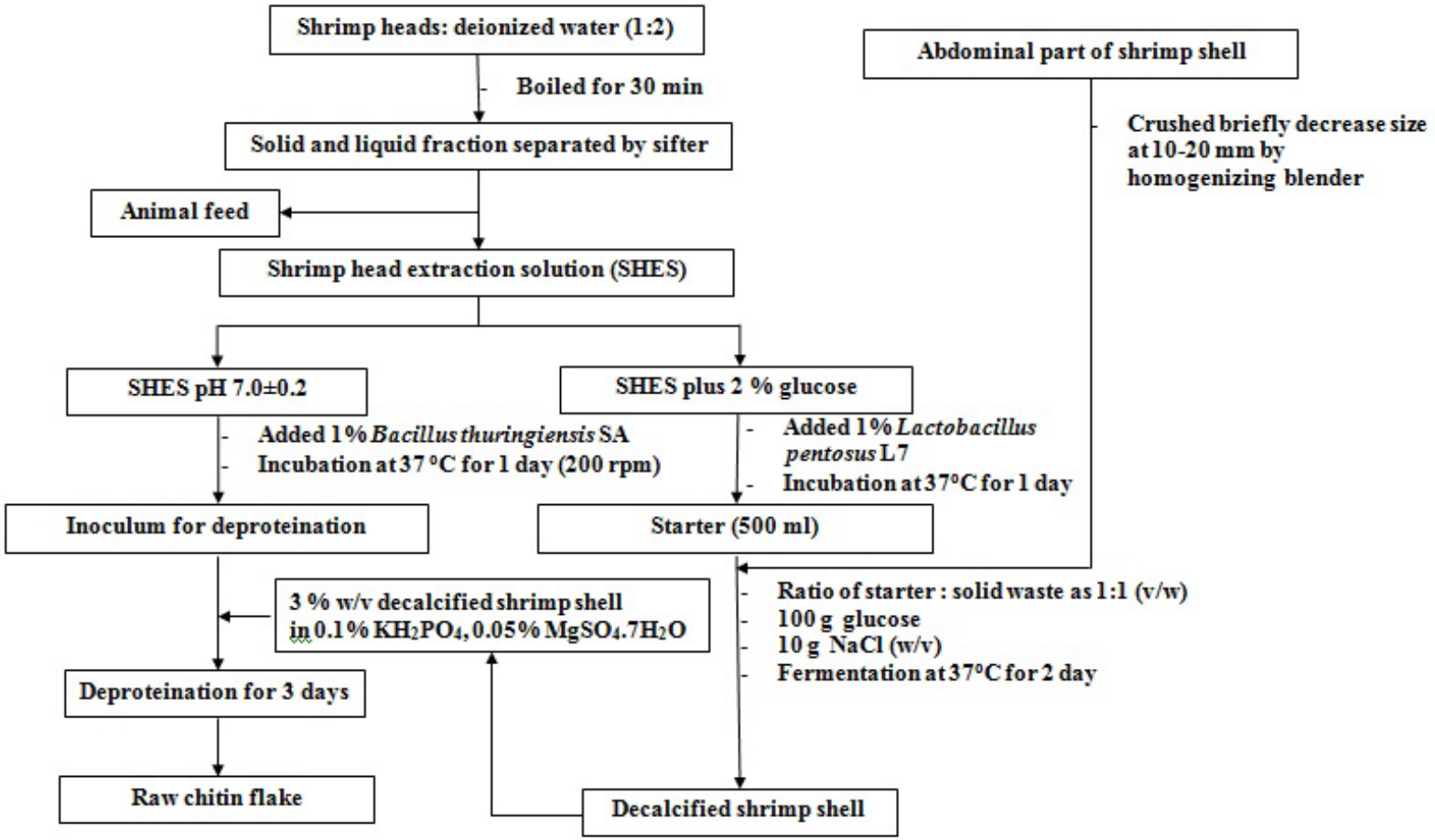


Figure 4.39 A two-step biological treatment process for chitin flake production from shrimp shells

4.15 Determination of Chitin Quality

4.15.1 Degree of Acetylation

Degree of acetylation on chitin samples were determined by UV-VIS Spectroscopy methods as described by Wu and Zivanovic (2008). The method was described in the "research methodology" (Section 3.3.15). Chitin sample was completely dissolved in concentrated phosphoric acid (85%) and was diluted for 100 times. Concentrated phosphoric acid was considered as a good solvent and the UV determination appears to be valid in the whole range of DA. Additionally, the results obtained by this method are well correlated with a solid state 13C NMR method, so far the best but the most expensive method for the DA analysis. However, polysaccharides generally undergo complex reactions under the conditions of concentrated acids and heat (Figure 4.40). For example, cellulose can be hydrolyzed into glucose and consequently converted into 5 hydroxymethylfurfural (HMF) and levulinic acid by acid catalyzed dehydration. One study has reported the formation of HMF from fully deacetylated chitosan after nitrous acid depolymerization. Apparently, the formation of HMF can introduce errors in the DA measurement because both acetyl-glucosamine and glucosamine can be converted to HMF. Intermediate glucofuranosyl oxazolinium ions of acetyl-glucosamine and similar products have been found in chitin solutions in concentrated phosphoric acid and anhydrous hydrogen fluoride. The intermediate ion is not stable and can be hydrolyzed into monosaccharide phosphate in diluted acids. Therefore, the solution was incubated at 60^{o}C for 2 h to convert the intermediate ion to monosaccharide phosphate (Figure. 4.40). The *N* acetyl glucosamine could absorb wavelength at 203 nm while glucosamine could not absorb. The concentration of glucosamine in diluted acid solution can be determined from equation (3.3).

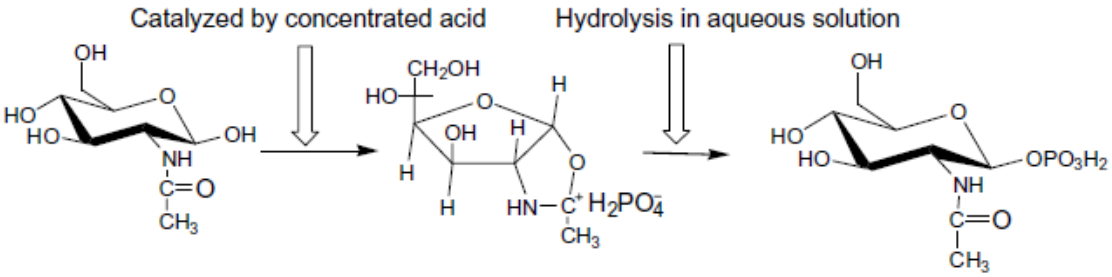


Figure 4.40 Formation and hydrolysis of glucofuranosyl oxazolinium ion from acetyl-glucosamine

Table 4.27 shows degree of acetylation of chitin flakes obtained from chemical, two steps biological processes and commercial chitin obtained from supplier in Samut Sakhon. The results revealed that chitin flakes obtained from biological process (98.16 ± 0.49% DA) gave the degree of acetylation higher than chemically processed chitin (96.45 ± 0.26% DA). In this point indicated that biological process could less damage to the structure of chitin.

Table 4.27 Degree of acetylation in chitin samples obtained by various processes

Chitin	Degree acetylation (% DA)
Commercial [A]	98.19±0.40[a]
Chemical process [B]	96.45±0.26[b]
Biological process [B]	98.16±0.49[a]

[A]; commercial chitin flake

[B]; chitin flake obtained from experiment with difference process

**Each value is expressed as mean ± s.d. (n = 3). Means with different letters within a column are significantly different (P < 0.05).

4.15.2 Color Properties

Table 4.28 shows color characteristics of commercial chitin, and experimental chitin obtained from chemical and biological process. The result revealed that chemically processed chitin were significantly lighter ($P < 0.05$) than biologically purified chitin (Table 4.28). L* value of 87.30 ± 1.60 and 84.71 ± 1.16 was determined from chemically processed and biologically purified chitins, respectively. In the other hands, chitins purified by biological process showed the highest redness (a* value is about 0.71 ± 0.20). However, b* value (7.18 ± 1.41) of biologically purified chitin was lower than commercial chitin. Three chitin samples showed a white property, leading to greater consumer preference and acceptability of the product. Figure 4.41 shows commercial chitin flakes.

Table 4.28 Color characteristics of commercial chitin, and experimental chitin obtained from chemical and biological processes

Chitin	Hunter color			
	L*	a*	b*	WI [C]
Commercial [A]	87.50 ± 0.83[a]**	0.09 ± 0.10[b]	9.31 ± 1.81[a]	84.41
Chemical process [B]	87.30 ± 1.60[a]	0.28 ± 0.12[b]	7.04 ± 0.96[b]	85.47
Biological process [B]	84.71 ± 1.16[b]	0.71 ± 0.20[a]	7.18 ± 1.41[b]	83.09

[A]; commercial chitin flakes

[C]; by calculation

[B]; chitin flakes obtained from experiment with difference process

**Each value is expressed as mean ± s.d. (n = 9). Means with different letters within a column are significantly different ($P < 0.05$).

Table 4.29 Properties of commercial chitin

Properties	Commercial chitin (Chemical process)
pH	8
Appearance	White and small of yellow
TKN (%w/w)	0.61 ± 0.15
Crude protein (%w/w)	1.16 ± 0.11
Ash (%w/w)	3.65 ± 0.36
Deproteinization efficacy (%)	-
Decalcification efficacy (%)	-

Each value is expressed as mean ± s.d. (n = 3).

Figure 4.41 Commercial chitin flakes

Table 4.29 shows properties of commercial chitin. Commercial chitin had high residue calcium (3.65 ± 0.36%) and protein (1.16 ± 0.11%) indicating incomplete extraction.

4.15.3 Shear Viscosity Analysis

The shear rate-dependent viscosity of chitin solutions was observed in Figure 4.42 which non-Newtonian behavior flow (Pseudoplastic) is observed as the rheological property of chitin solution. Biologically purified chitin showed viscosity higher than chemically processed chitin and commercial chitin. Viscosities of biologically purified chitin, chemically processed chitin, and commercial chitin were 110 ± 15.13, 82 ± 13.56, and 77 ± 19.28 mPa.s, respectively (Table 4.30). The viscosity of chitin depends on extraction process and quality of chitin at the end of process which remaining of high calcium in commercial chitin (3.65 ± 0.36% ash content) leading to lower viscosity. Viscosity of chitin involves intrinsic properties of chitin which it can be used to determine the chitin quality. The biological process could preserve the structure of shrimp chitin better than the chemical process by retaining higher DA and viscosity.

In addition, the increasing of temperature influenced significant decreasing of appearance viscosity (η_{app}). The relationship between shear viscosity at constant shear rate (30 s^{-1}) and temperature for chitin solution with concentration of 0.1% is plotted in Figure 4.43 (R^2 = 0.9838). The viscosity value at difference temperature obtained from a constant shear rate can be correlated with temperature according to the Arrhenius equation (4.1):

$$\eta = A.e^{-Ea/RT} \tag{4.1}$$

Table 4.30 Appearance viscosity of chitin samples

Chitin products	Viscosity (mPa.s)
Biological	110 ± 15.13
Chemical	82 ± 13.56
Commercial	77 ± 19.28

Viscosity was determined at shear rate of 30 s^{-1} at 25 ^{o}C.

Each value is expressed as mean ± s.d. (n = 6).

Table 4.31 Herschel-Bulkley model for chitin solution

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Chitin sample	Temperature (^oC)	Herschel-Bulkley equation	R^2
Commercial	25	Log (σ) =2.0475+0.7132 Log (γ)	0.9875
Chemical	25	Log (σ) =2.3525+0.6445 Log (γ)	0.9960
Biological	25	Log (σ) =2.3942+0.6304 Log (γ)	0.9921
	35	Log (σ) =2.3664+0.6323 Log (γ)	0.9887
	45	Log (σ) =2.2896+0.6398 Log (γ)	0.9904
	55	Log (σ) =2.1708+0.6632 Log (γ)	0.9911

where A is a constant related to molecular motion, Ea is the activated energy for viscous flow at constant shear rate, R is the universal gas constant and T is the absolute temperature in K. Figure 4.44 represents an Arrhenius plot for chitin solution with concentration of 0.1%. A linear relationship (R^2= 0.9553) was obtained from Arrhenius plot with apparent activation energy of 10.79 kJ mol^{-1}. Figure 4.45 shows the effect of shear rate (γ) on shear stress (σ), shear stress increased with increasing of shear rate while at the same shear rate, shear stress was lower at the high temperature. From the figure 4.45, it is clear that chitin solution showed pseudoplastic none Newtonian behavior.

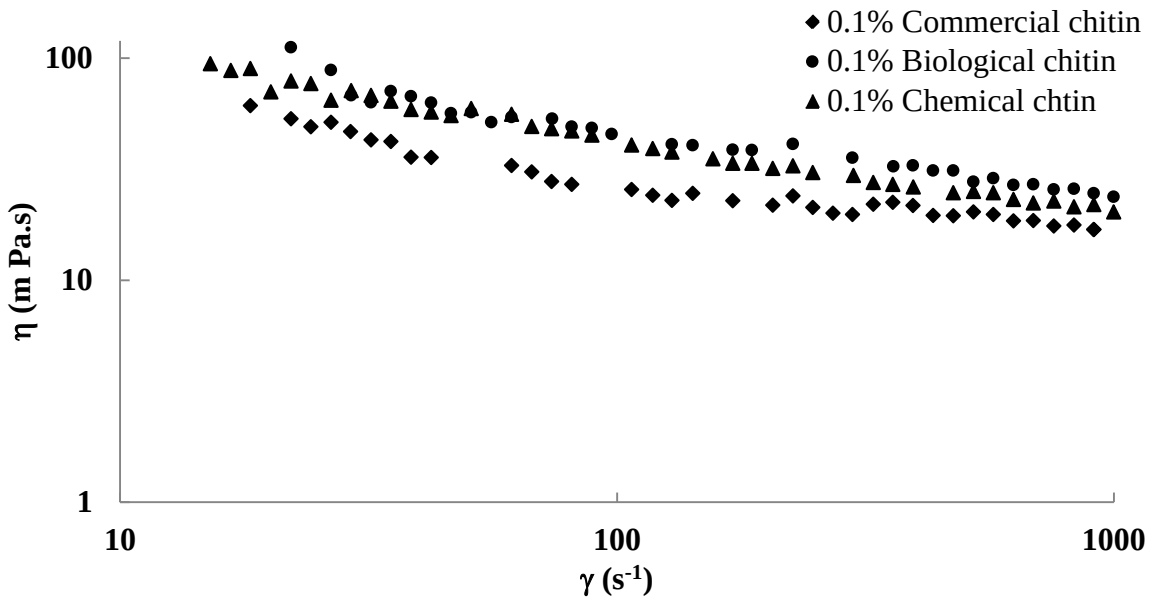

Figure 4.42 Influence of shear rate on the rheological curves of chitin samples dissolved by dimethylacetamide containing 5% $LiCl_2$ at 25^{o}C

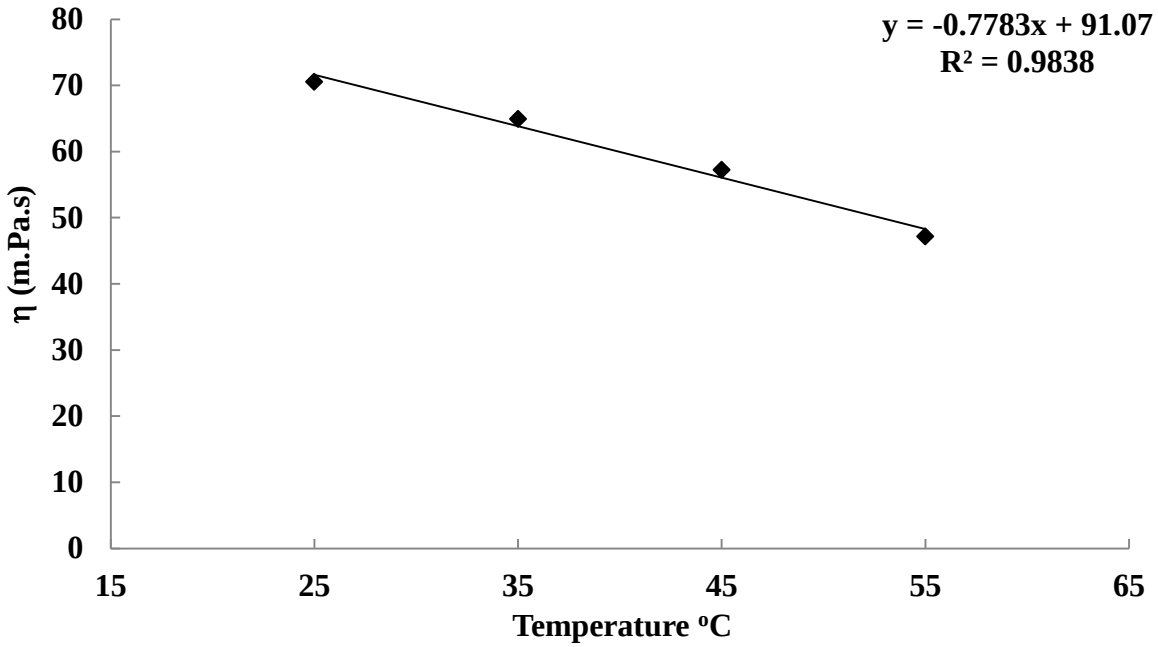

Figure 4.43 The effect of temperature on viscosity of chitin solution at a constant shear rate of 30 s^{-1}

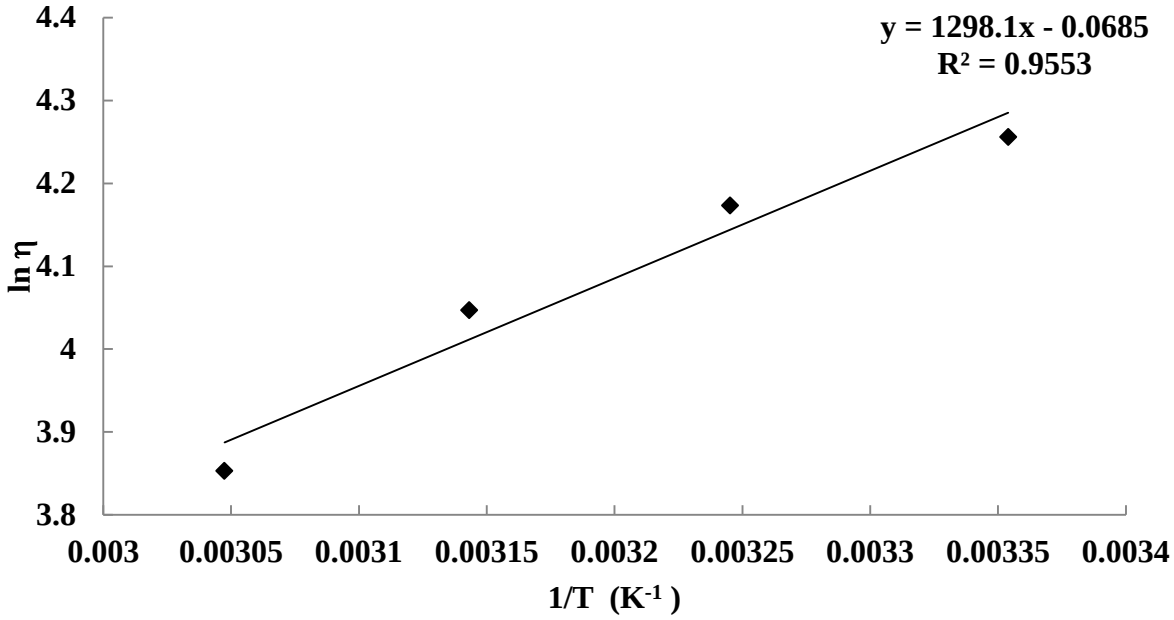

Figure 4.44 The Arrhenius plot of ln η versus 1/T for chitin solution

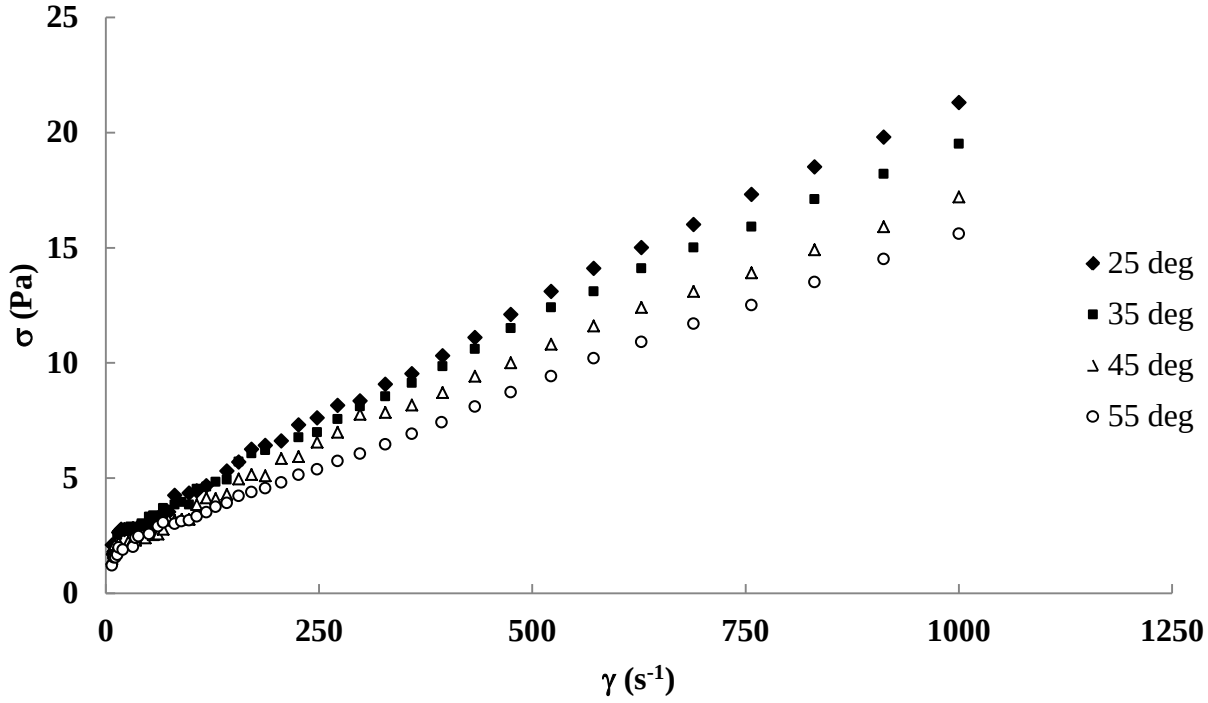

Figure 4.45 Shear stress of chitin solution at different range of temperature versus shear rate

4.16 Chitosan Production

Chitosan is a name of de - N - acetylated chitin chain, poly-(β-(1-4)-linked-2-amino-2-deoxy-D-glucose. Generally, chitosan requires at least 70% of degree of deacetylation (DA). Commercial chitosan has been produced by deacetylation of chitin in 30 – 60% sodium or potassium hydroxide solutions at 80 - 140^{o}C. In this study, 50% NaOH at 121^{o}C was selected as condition to prepare chitosan flakes. Characteristics of chitosan products in terms of viscosity and degree of deacetylation depend on deacetylation condition and initial quality of "pre-product" chitin. In conventional chitin purification, chitin is usually isolated by a simple process which involves the use of alkaline and acid to deproteinize and decalcify the shells. Disadvantages of conventional method are the requirement for a high amount of water and energy and the release of large effluent streams containing concentrated corrosive acid and alkaline residues. In addition, the strong condition during chitin purification makes damage to an intensive chitin structure which has a negative effect on quality of chitosan after deacetylation. To overcome the disadvantages of conventional methods, a novel process for chitosan production was investigated in this study. Qualities of chitosan prepared from chitin extracted by difference processes were investigated (Figure 4.46).

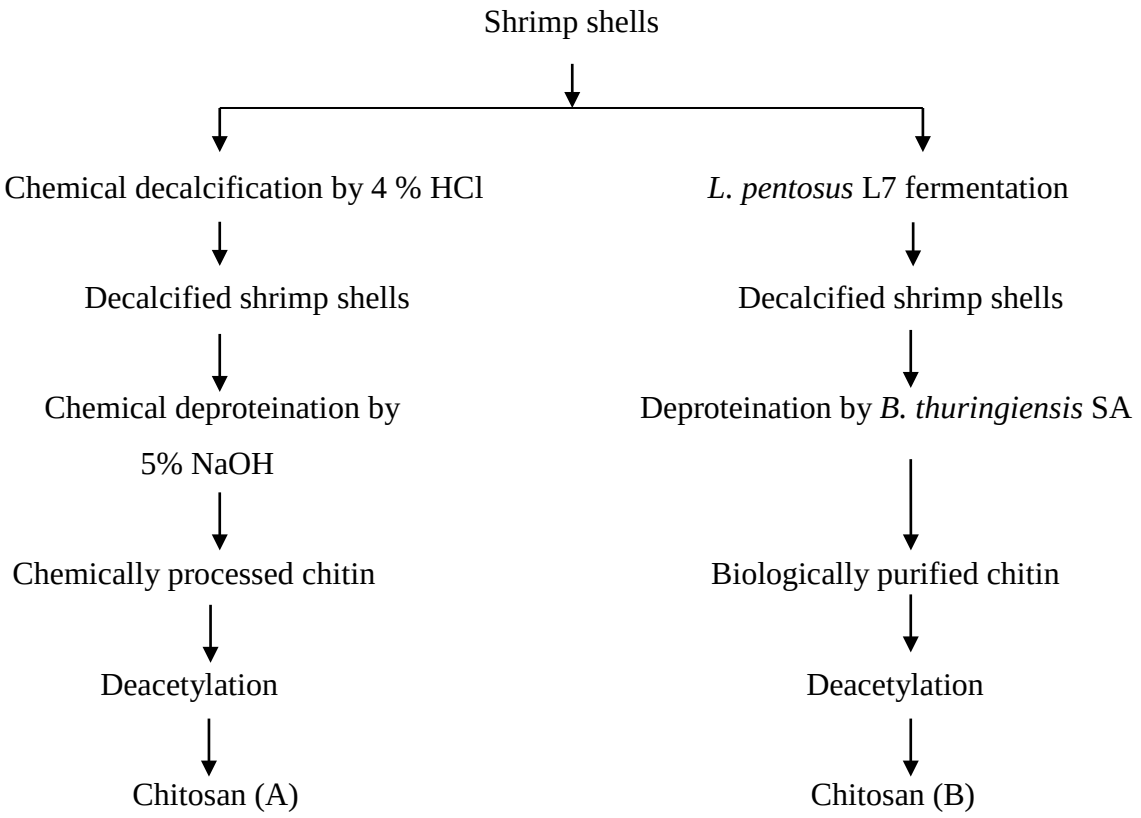

Figure 4.46 Production of chitosan from chitin prepared from different processes; chemically processed chitin (A), and biologically processed chitin (B)

4.16.1 Production of Chitosan from Chemically Processed Chitin

In the section 4.14.1, chemically processed chitin was prepared. The properties of chemically processed chitin are shown in Table 4.21. Chitin can be converted to chitosan by deacetylation with concentrated NaOH under strong condition. The temperature for deacetylation of chitin used in this study was 50% NaOH at 121^{o}C. The results showed that deacetylation time at 5 h could deacetylate chitin up to 82.23%, whereas the degree of deacetylation in the treatments 2, 3, and 4 h were less than 70% indicating that chitosan was obtained when deacetylation time was higher than 5 h (Table 4.32). The residual ash and protein contents in every treatment were lower than 1 and 0.5%, respectively; a good chitosan quality was obtained (Table 4.32).

Table 4.32 The effect of deacetylation time on chitosan properties prepared from chemically processed chitin

Deacetylation time (h)	Degree of deacetylation (%)	Residue ash (%)	Residue protein (%)	Viscosity (mPa.s)
2	28.64 ± 0.87	0.25 ± 0.19	0.25±0.20	22.7±0.91
3	57.69 ± 0.48	0.20 ± 0.23	0.23±0.19	24.9±0.96
4	66.43 ± 0.54	0.28 ± 0.22	0.24±0.20	28.4±1.53
5	82.23 ± 1.16	0.25 ± 0.14	0.25±0.18	323 ±15.6
6	94.18 ± 0.65	0.23 ± 0.14	0.27±0.14	170.3±15.3

Viscosity (η) was determined at shear rate of 50 s^{-1} and 20 ^{o}C.

Each value is expressed as mean ± s.d. (n = 3).

Figure 4.47 Chitosan flakes obtained from deacetylation of chemically processed chitin at 121^{o}C for 5 h

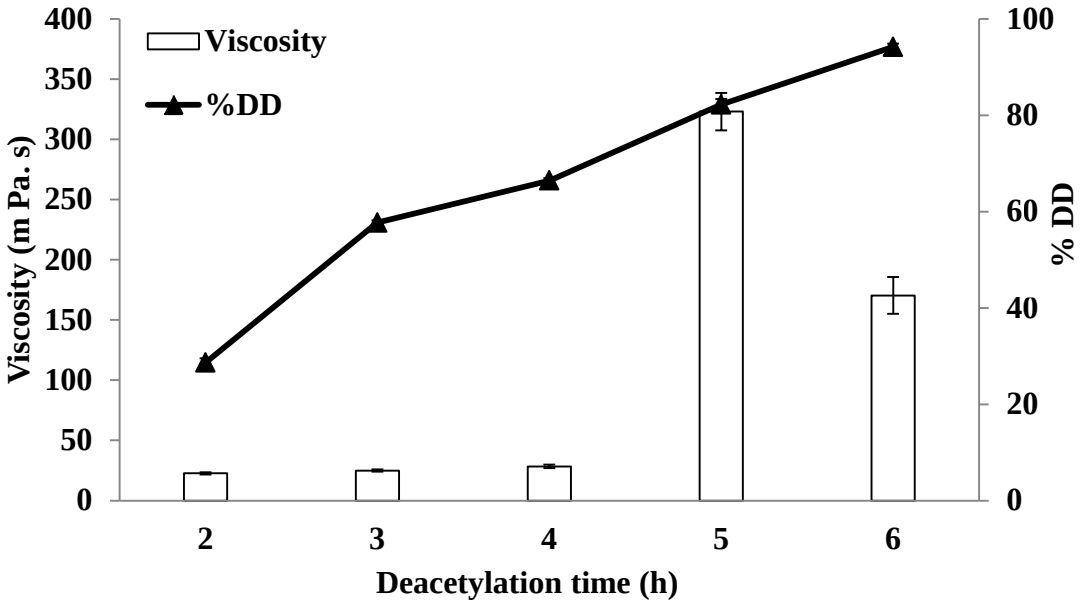

Figure 4.48 Viscosity (mPa.s) and degree of deacetylation (%DD) of chitosan samples prepared form chemically processed chitin deacetylated at 121^{o}C by increasing reaction time

When reaction time for deacetylation increased from 5 h to 6 h, viscosity of chitosan (170.3 ± 15.3 mPa.s) decreased. In this point indicated that chitosan polymer was fragmented. Therefore, deacetylation of chemically processed chitin at 121^{o}C for 5 h gave a highest viscosity (323 ± 15.6 mPa.s). Figure 4.48 represents viscosity (mPa.s) and degree of deacetylation (%DD) of chitosan samples prepared form chemically processed chitin deacetylated at 121^{o}C by increasing reaction time.

Table 4.33 The effect of deacetylation time on color characteristics of chitosan prepared from chemically processed chitin

Deacetylation time (h)	Hunter color			
	L*	**a***	**b***	**WI****
2	78.75±0.03^{a}	0.77±0.01^{b}	8.35±0.12^{ab}	77.15
3	76.97±0.58^{b}	0.57±0.04^{cd}	8.00±0.20^{b}	75.61
4	76.80±0.10^{b}	0.62±0.03^{bc}	7.28±0.05^{c}	75.67
5	73.08±1.97^{d}	0.44±0.06^{d}	7.03±0.33^{c}	72.13
6	74.92±0.65^{c}	1.42±0.34^{a}	8.64±1.21^{a}	73.43

**; by calculation

Each value is expressed as mean ± s.d. (n = 9). Means with different letters within a column are significantly different ($P < 0.05$).

Table 4.33 shows color characteristics of chitosan prepared from chemically processed chitin deacetylated at 121^{o}C by increasing reaction time. The results showed that increase of reaction time from 2 h to 5 h decreased L* value from 78.75 ± 0.03 to 73.08 ± 1.97. White index (WI) of final chitosan deacetylated at 121^{o}C for 5 h was 72.13. However, chitosan sample showed white property leading to the preference and acceptability of consumers to product. Figure 4.47 shows chitosan flakes obtained from deacetylation of chemically processed chitin at 121^{o}C for 5 h.

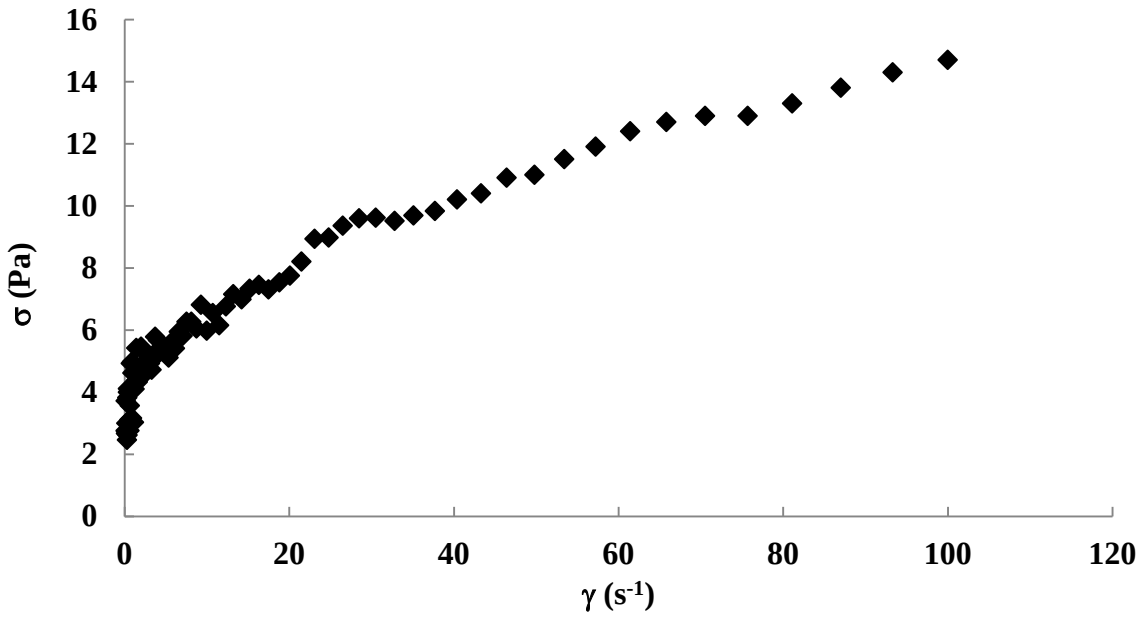

Figure 4.49 Plot of shear stress (σ) of 1% chitosan solution versus shear rate (γ)

The shear rate-dependent viscosity of 1% chitosan solution was observed in Figure 4.49 which non-Newtonian behavior flow (Pseudoplastic) is observed as the rheological property of 1% chitosan solution. Pseudoplastic fluid property was observed. The increase of shear rate leads to increasing shear stress (σ), while viscosity decreased (Figure 4.49 and 4.50). From Herschel-Bulkley plot, linear equation; Log (σ) =3.6135+0.2364 Log (γ) was observed (Figure 4.51) leading to n value of 0.2364 was calculated from the equation; n value is less than 1 which it is major characteristic of pseudoplastic fluid. Pseudoplastic behavior of 1% chitosan solution was clearly confirmed by Herschel-Bulkley plot (Figure 4.51).

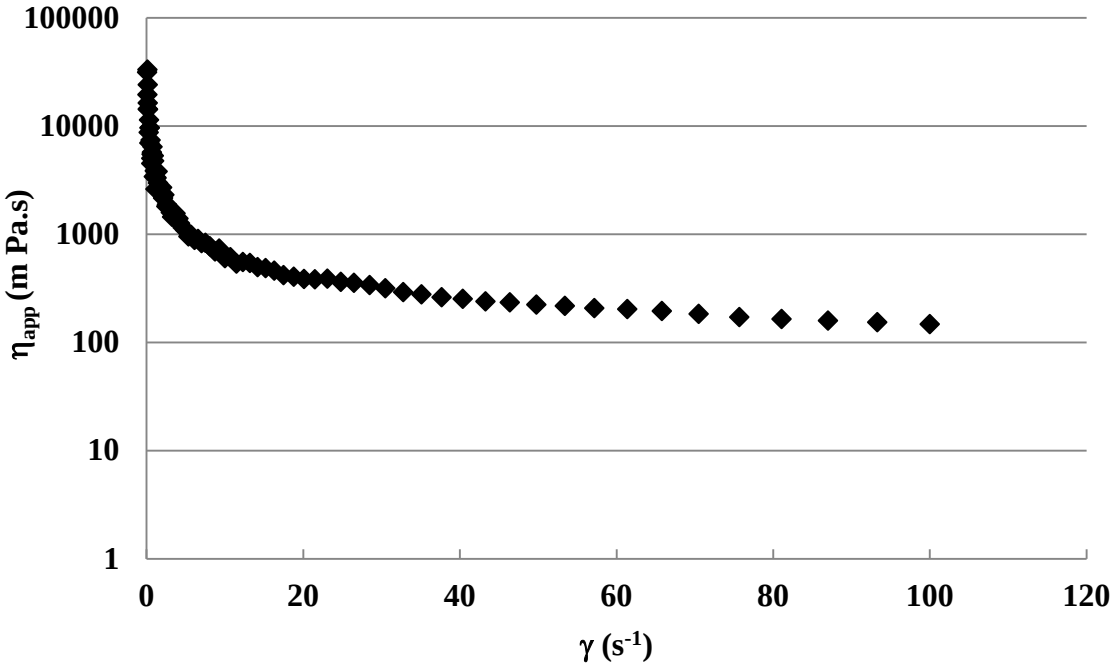

Figure 4.50 Plot of viscosity (η_{app}) of 1% chitosan solution versus shear rate (γ)

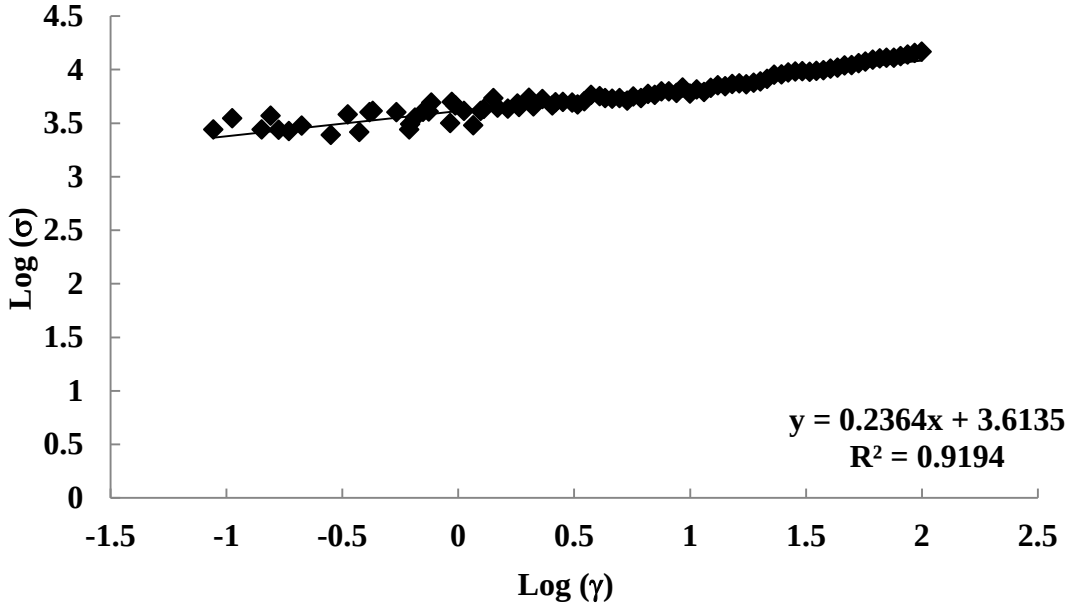

Figure 4.51 The Herschel-Bulkley plot of Log σ versus Log γ for 1% chitosan solution

4.16.2 Production of Chitosan from Biologically Purified Chitin

Chitin obtained from the section 4.14.5 was deacetylated at 121^{o}C by increasing reaction time from 2 h to 6 h. The properties of chitosan obtained by deacetylation of biologically purified chitin are shown in Table 4.34. Under the selected deacetylation temperature, deacetylation time at 5 h could convert chitin to chitosan; degree of deacetylation up to 83.18 ± 1.54% was observed (Table 4.34). The ash and protein residues were lower than 2 and 0.5%, respectively in every treatments; a good chitosan quality was obtained (Table 4.34). Figure 4.52 shows chitosan flakes obtained from deacetylation of biologically purified chitin (Deacetylation for 5 h).

Table 4.34 The effect of deacetylation time on chitosan properties prepared from biologically purified chitin

Deacetylation time (h)	**Degree of deacetylation (%)**	**Residue ash (%)**	**Residue protein (%)**	**Viscosity (mPa. s)**
2	31.33 ± 1.00	1.44 ± 0.36	0.4 ± 0.12	46.8 ± 7.45
3	50.83 ± 2.31	1.52 ± 0.29	0.34 ± 0.10	65.7 ± 7.00
4	59.63 ± 1.18	1.57 ± 0.35	0.39 ± 0.24	139 ± 17.34
5	83.18 ± 1.54	1.58 ± 0.55	0.39 ± 0.26	1007 ± 14.73
6	90.11 ± 0.64	1.36 ± 0.50	0.34 ± 0.26	189 ± 26.66

Viscosity (η) was determined at shear rate of 50 s^{-1} and 20 ^{o}C.

Each value is expressed as mean ± s.d. (n = 3).

Figure 4.52 Chitosan flakes obtained from deacetylation of biologically purified chitin at 121^{o}C for 5 h

Table 4.35 The effect of deacetylation time on color characteristics of chitosan prepared from biologically purified chitin

Deacetylation time (h)	**Hunter color**			
	L*	**a***	**b***	**WI****
2	78.13±2.05^{a}	0.80±0.56^{b}	6.52±0.68^{b}	77.16
3	76.02±1.69^{b}	0.31±0.11^{c}	4.74±0.40^{c}	75.55
4	78.49±0.56^{a}	0.89±0.08^{ab}	7.05±0.15^{b}	77.34
5	77.29±1.42^{ab}	0.70±0.22^{b}	6.41±0.58^{b}	76.39
6	76.98±1.35^{ab}	1.11±0.09^{a}	8.34±1.16^{a}	75.49

**; by calculation

Each value is expressed as mean ± s.d. (n = 9). Means with different letters within a column are significantly different ($P < 0.05$).

Table 4.35 shows color characteristics of chitosan prepared from biologically purified chitin deacetylated at 121^{o}C by increasing reaction time. The results showed that the

increasing of reaction time from 2 h to 5 h, L* value significantly decreased from 78.13 ± 2.05 to 77.29 ± 1.42. White index (WI) of final chitosan deacetylated at 121^{o}C for 5 h was 76.39. However, chitosan sample showed white property leading to the preference and acceptability of consumers to product.

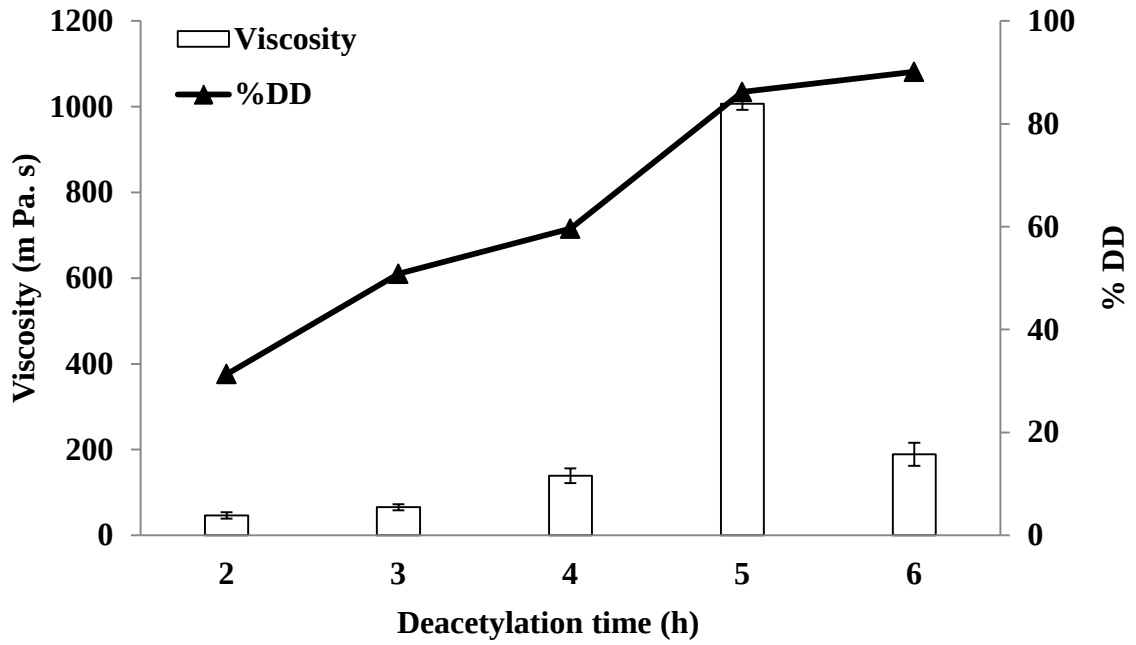

Figure 4.53 Viscosity (mPa.s) and degree of deacetylation (% DD) of chitosan sample prepared form biologically purified chitin deacetylated at 121^{o}C by increasing reaction time

When reaction time for deacetylation increased from 5 h to 6 h, viscosity of chitosan (189 ± 26.66 mPa.s) decreased due to chitosan polymer was fragmented. Therefore, deacetylation of biologically purified chitin at 121^{o}C for 5 h gave highest viscosity (1007 ± 14.73 mPa.s). Figure 4.53 represents viscosity (mPa.s) and degree of deacetylation (% DD) of chitosan samples prepared form biologically purified chitin deacetylated at 121^{o}C by increasing reaction time.

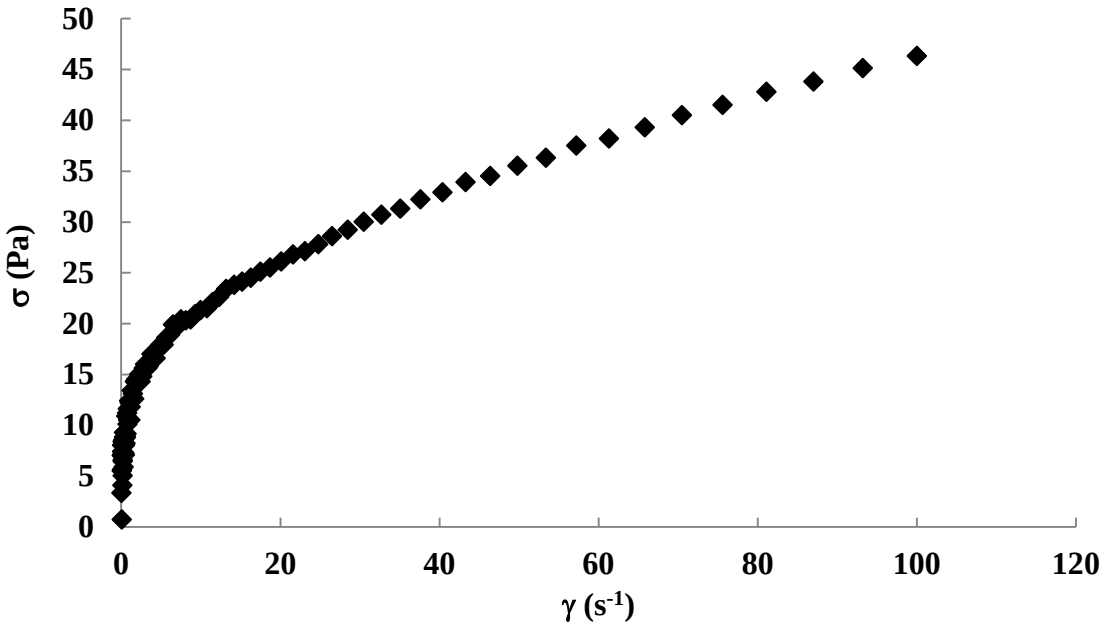

Figure 4.54 Plot of shear stress (σ) of 1% chitosan solution versus shear rate (γ)

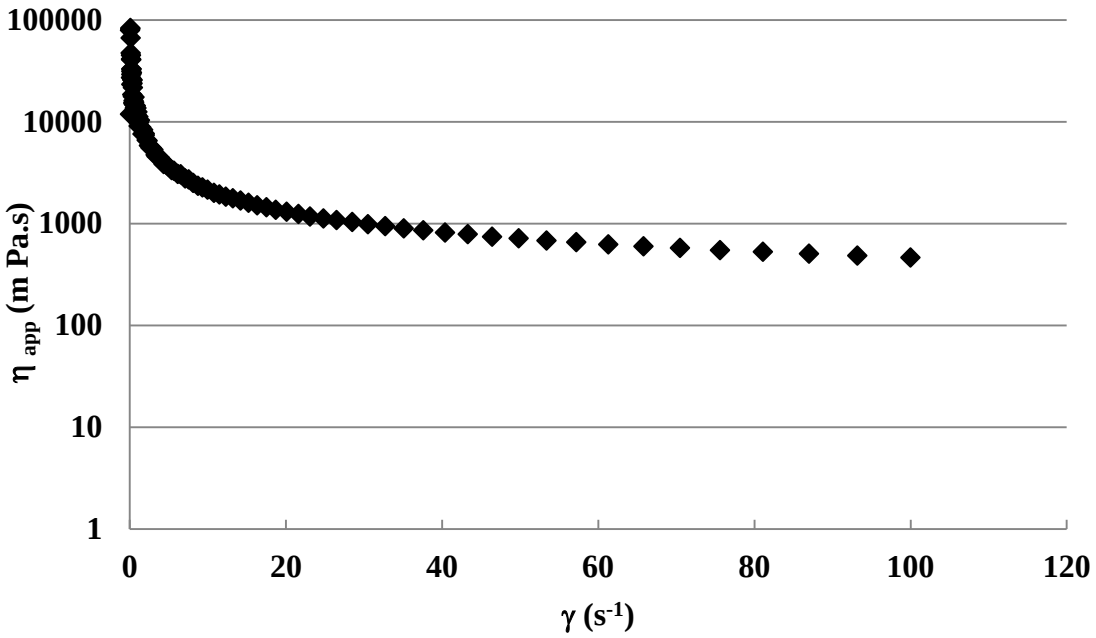

Figure 4.55 Plot of viscosity (η_{app}) of 1% chitosan solution versus shear rate (γ)

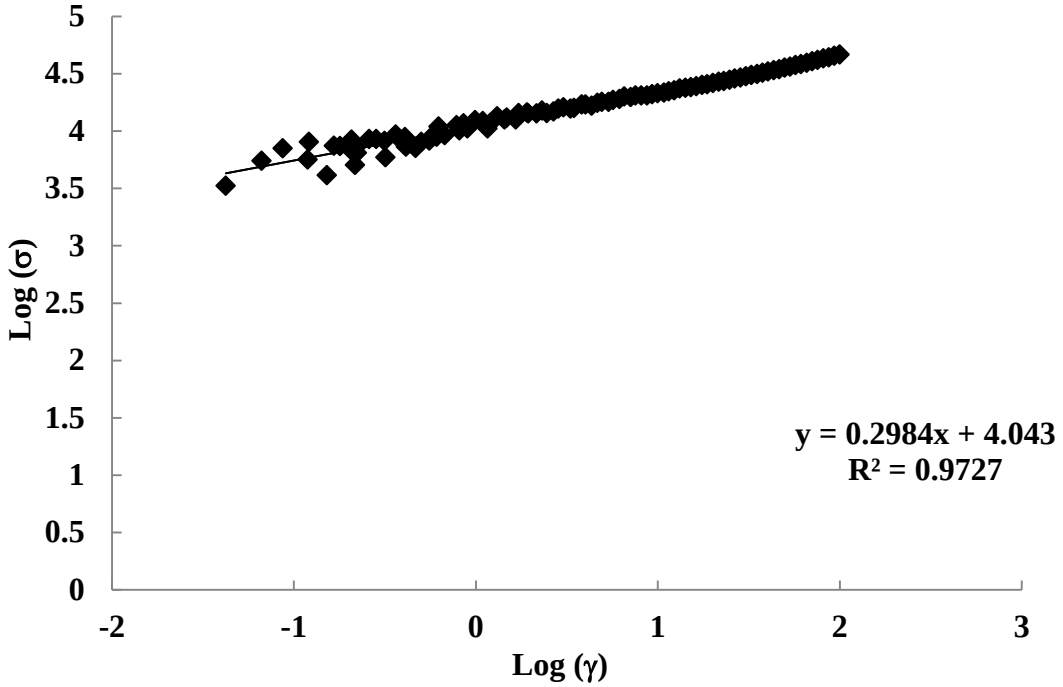

Figure 4.56 The Herschel-Bulkley plot of Log σ versus Log γ for 1% chitosan solution

The shear rate-dependent viscosity of 1 % chitosan solution obtained from deacetylation of biologically purified chitin was observed in Figure 4.54 which non-Newtonian behavior flow (Pseudoplastic) is observed as the rheological property of 1% chitosan solution. Pseudoplastic fluid properties was observed when increasing of shear rate leads to increasing shear stress (σ), while viscosity was decreased (Figure 4.54 and 4.55). From Herschel-Bulkley plot, linear equation; Log (σ) =4.043+0.2984 Log (γ) (R^2=0.9727) was determined (Figure 4.56) leading to n value of 0.2364 was calculated from the equation; n value is less than 1 which it is major characteristic of pseudoplastic fluid. Pseudoplastic behavior of 1% chitosan solution was clearly confirmed by Herschel-Bulkley plot (Figure 4.56).

CHAPTER 5 CONCLUSION

The crustacean waste from seafood industry plays an important role as a source for chitin and chitosan preparations. In this study, shrimp shells (Abdominal part) contained 27.92 ± 0.94% ash, 2.37 ± 0.95% lipid, and 25.92 ± 2.59% crude protein, whereas shrimp heads had crude protein, ash, and lipid contents at 40.74 ± 2.66%, 22.21 ± 0.61%, and 13.87 ± 1.12%, respectively. The shrimp shells had less protein and lipid than shrimp head; therefore, abdominal parts were the good raw material for chitin and chitosan preparations.

Commercial chitin and chitosan are prepared by treating crustacean shells with chemical processes using acid and alkaline to eliminate calcium and protein from the shells. Liquid wastes derived from these chemical processes have produced high impact environmental pollution. Biological processes for production of chitin from shrimp shells were investigated as potential alternative to the chemical treatment processes. To deproteinize shrimp shells, the 14 isolates of proteolytic bacteria were isolated from mangrove soil, Bangkhuntien by using 1% skim milk agar. Among them, the bacterial isolate SA was found as the effective producer of proteases specific for shrimp shell protein. The bacterial isolate SA was tested for deproteinization of shrimp shells. Deproteinization efficacy up to 90.22% was obtained after 4 days indicating that the replacement of alkaline extraction by microbial deproteinization was possible.

The selected isolate SA was identified to be *Bacillus thuringiensis* SA based on Gry B gene and the present of parasporal crystal body inside SA cells during sporulation stage. Enzyme characterizations were performed to observe the effects of temperature and pH on the activity of protease produced by *B. thuringiensis* SA. The optimum temperature and pH on activity were determined at 50^{o}C and pH 7, respectively. EDTA was found that only has a significant effect on activity indicating that protease was a neutral metalloprotease. Enzyme purification was done based on chromatography techniques. Analysis of the purified enzyme by 10% SDS-PAGE revealed that a single band with a molecular mass of about 35 kDa was determined.

To remove calcium carbonate form shrimp shells, acid producing bacteria were isolated from Nham, Thai traditional fermented sausage. There were 19 acid producing bacterial isolates obtained from 8 Nham samples. All isolates were tested for acid production efficacy in MRS broth. The isolate L7 which showed the highest acid production efficacy was selected to ferment shrimp shells. The fermentation of shrimp shells by isolate L7 was performed by using fresh shrimp shells waste. The various types of carbon sources (glucose, whey, sucrose and molasses) were supplemented to investigate the effect of carbon sources on decalcification efficacy. The highest decalcification efficacy up to 97% was determined when 20% glucose (w/w $_{\text{shrimp shell weight}}$) was supplemented; therefore, glucose was the best carbon sucrose for decalcification of shrimp shells. The bacterial isolate L7 was identified to be *Lactobacillus pentosus* L7.

The high price of bacterial mediums for cultivation of *Lactobacillus pentosus* and *Bacillus thuringiensis* could overcome by SHES obtained from boiling the shrimp heads. The SHES could not support the growth of *L. pentosus* L7 unless carbon source such as glucose was supplemented. SHES plus 2% glucose is much cheaper than the traditional culture medium for lactobacilli; MRS medium. Therefore, SHES plus 2% glucose medium would be used as cultivation medium to prepare the inoculum for lactic acid fermentation of shrimp shells. For *B. thuringiensis* SA, SHES, NB, and TSB gave the similar growth support for the bacteria as indicating SHES would be used as culture medium for *B. thuringiensis* SA.

The one step biological purification could not completely extract protein and calcium in shrimp shells. The residual crude protein and calcium in decalcified or deproteinized shrimp shells led to low quality of the final product. A two-step purification of the 1st step with the *L. pentosus* L7 and the 2nd step with *B. thuringiensis* SA for decalcification and deproteinization of shrimp shells was demonstrated. For the first step, *L. pentosus* L7 fermentation was used for decalcification of shrimp shell. The fermentation was completed in 48 h. After fermentation, the amounts of residual calcium in the form of ash (1.44 ± 0.41%) and crude protein (23.24 ± 2.50%) were further eliminated by the activity of proteolytic *B. thuringiensis* SA. After decalcification and deproteinization of the shrimp shells, residual calcium, crude protein and % DA of shrimp chitin flakes were 1.72 ± 0.37%, 3.80 ± 1.28% and 98.16 ± 0.49%, respectively. The chitin flakes made from same lot of shrimp shells using chemical

process extraction had residual calcium, crude protein content and % DA at 0.57 ± 0.14 %, 0.34 ± 0.10% and 96.45%, respectively. The viscosity of biologically purified chitin was 110 ± 15.1 mPa.s, whereas the viscosity of chemical processed chitin was 82 ± 13.6 mPa.s. This point indicated that the biological process caused less damage to the intensive of chitin structures than chemical process, demonstrated by the higher degree of acetylation and viscosity of the product.

Chitosan can be prepared by deacetylation of chitin with 50% NaOH at 121°C for 5 h. The viscosity of chitosan prepared from chitin extracted by two-step biological process was 1,007 ± 14.7 mPa.s, whereas chitosan prepared from chitin made from the same lot of shrimp shells using a chemical extraction process had a viscosity of 323 ± 15.6 mPa.s, indicating that biologically purified chitin gave chitosan with a high quality.

In summarization, the important value added bio-product from shrimp shells is chitin which can be deacetlylated to chitosan. The viscosity and intensive structure of final chitosan product strongly depended on the properties of “pre-product chitin”. Chitosan with a high viscosity could be prepared by this process.

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APPENDIX A

APPROXIMATE ANALYSIS

1. Ash Content

Dry ashing procedures use a high temperature muffle furnace capable which temperature is maintained at 500-600^{o}C. Water and other volatile materials are vaporized and organic substances are burned in the presence of the oxygen in air to CO_2, H_2O and N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates. Although most minerals have fairly low volatility at these high temperatures, some are volatile and may be partially lost, e.g., iron, lead and mercury.

Apparatus

- Porcelain crucibles or dashes.
- Electric muffle furnace, capable of maintaining a temperature of 575 ± 25°C. Ensure enough oxygen is admitted to the furnace chamber to complete removal of the carbonaceous material.
- Desiccator.

Procedure

- A crucible was put and burn in the muffle furnace at 575 ± 25°C for 12 h.
- The weight of crucibles was recorded.
- Sample of 0.5 to 2.0 g was weighted into crucibles.
- Crucibles contained samples was burn in the muffle furnace at 575+25°C for 12 h.
- After cooling at room temperature, the weight of crucibles plus ash was recorded.
- % Ash content was determined according to equation (1).

$$\%Ash = \frac{Weight_{crucible\ plus\ ash} - Weight_{crucible}}{DW_{sample}} \times 100 \quad (1)$$

When

DW_{sample} = dried weight of sample

2. Determination of Crude Fat Content by Soxhlet Extraction

Apparatus

1. Soxhlet extractor
2. Oven for elimination of solvent
3. Petrolium ether
4. Membrane filter
5. Cellulose extraction thimbles

Procedure

- Soxhlet beaker and membrane filter were put in oven at 80^{o}C for overnight to obtain constant weight.
- The weight of Soxhlet beaker was recorded.
- Sample (5.0 g) was weighted into membrane filter.
- The membrane filter contained sample was put into cellulose extraction thimbles.
- Cellulose extraction thimbles were set in Soxhlet extractor.
- Soxhlet beaker containing Petrolium ether were also set in Soxhlet extractor.
- Soxhlet extractor was operated at 110^{o}C for 6 h.
- After extraction, residual Petrolium ether was removed by oven at 80^{o}C for overnight.
- The weight of Soxhlet beaker plus fat was recorded.
- % Ash content was determined according to equation (2).

$$\%Fat = \frac{Weight_{Soxhlet\ beaker\ plus\ fat} - Weight_{Soxhlet\ beaker}}{DW_{sample}} \times 100 \quad (2)$$

When

DW_{sample} = dried weight of sample

3. Determination of Crude Protein Content by Total Kjeldahl Nitrogen or TKN

Reagents and chemical

1. Concentrated sulfuric acid
2. Catalyst
3. 40 % Sodium hydroxide (40% NaOH)
4. 1N Sodium hydroxide (1N NaOH)
5. 4 % Boric acid
6. Mixed indicator
7. Ethanol
8. Hydrochloric (HCl)
9. Sodium carbonate (Na_2CO_3)
10. Copper sulfate ($CuSO_4.5H_2O$)
11. Potassium sulfate (K_2SO_4)

Chemical preparation

1. Catalytic powder

    Mixing 0.5 g copper sulfate ($CuSO_4.5H_2O$) with 10 g potassium sulfate (K_2SO_4)

2. 40% Sodium hydroxide (40% NaOH)

    Dissolving 400 g sodium hydroxide by distilled water, adjust to 1,000 ml

3. 1N Sodium hydroxide (1N NaOH)

    Dissolving 40 g sodium hydroxide by distilled water, adjust to 1,000 ml

4. 4% Boric acid

    Dissolving 4 g Boric acid in 100 ml distilled water

5. Mixed indicator

Mixing 0.01 g bromocresal green with 0.02 g methylred and then dissolving by 10 ml 95% ethanol

Procedure

Digestion

1. 0.5-1 g Sample is weighted and put into digestion tube.
2. Catalyst is added and then 25 ml conc. H_2SO_4 is added.
3. Sample is digested at 420^{o}C until solid disappeared.

Distillation

1. 150 ml Distilled water was added into digestion tube and then 50 ml 40% NaOH is Added.
2. Digestion tube was connected to digestion machine; outlet was dripped into erlenmeyer flask containing 25 ml Boric acid (4%).
3. Mixed indicator was dropped (2-3drops)
4. Distillation was done for 5 min (Stream is set at 100%)

Titration

1. Sample was titrated with standard 0.1 N HCl solutions until pink solution was obtained.
2. Deionized water was used as the blank.

Calculation

$$\text{Nitrogen (\%)} = \frac{1.4 \times 0.1 \times (V_s - V_b)}{W_s} \quad (4)$$

$$\text{Protein (\%)} = \text{\% Nitrogen} \times \text{Conversion factor} \quad (5)$$

When

V_s = Volume of 0.01N NaOH used for titration of sample (ml)

V_b = Volume of 0.01N NaOH used for titration of blank (ml)

W_S = Sample weight (g)

Conversion factor = 6.25

Determination of standard solution of HCl

1. 5 g Na_2CO_3 was weighted into Blender to prepare powder.
2. Na_2CO_3 powder was put in oven at 265°C for 1 h and kept in Desicator until used.
3. 0.13 g dehydrated Na_2CO_3 was put into flask containing 20 ml distilled water and mixed.
4. Mixed indicator was added for 5 drops.
5. Sample was titrated with HCl until pink solution is obtained (A1).
6. Solution was boiled for 2-3 min and cooled at room temperature.
7. Sample was titrated again with HCl until pink solution is obtained (A2).
8. Concentration of HCl was calculated by equation (6).

$$\text{HCL (mol/l)} = \frac{2000 \times Weight\ of\ Na_2CO_3}{Molecular\ weight\ of\ Na_2CO_3 \times (A_1 - A_2)} \quad (6)$$

4. Determination of Carbohydrate Content

Carbohydrate content was determined according to equation (7);

Carbohydrate content = 100%- Protein content (%)-Ash content (%)- Fat content (7)

5. Determination of Fat by Solvent Extraction

Reagent

1. Hexane

Apparatus

1. Separatory funnel
2. Oven for elimination of solvent
3. Flask

Procedure

- Lipid sample (10 ml) was put into 250 ml flask (record used volume).
- 100 ml hexane is added.
- The fat extraction was done at room temperature (20^{o}C) by constant stirrer for 24 h.
- Mixture solvent was separated by Separatory funnel and put into new flaks (know the weight).
- Remove solvent (hexane) by oven at 80^{o}C for overnight.
- The weight of flask plus fat was recorded.
- % Fat (w/v) in sample was determined according to equation (8).

$$\%Fat = \frac{Weight_{flask\ plus\ fat} - Weight_{flask}}{V_{sample}} \times 100 \quad (8)$$

APPENDIX B

STANDARD CURVES

1. Reducing Sugar Analysis (Nelson, 1944)

Reagents

1. Na_2SO_4 (anhydrous)
2. $NaCO_3$
3. $NaHCO_3$
4. $CuSO_4.5H_2O$
5. Ammonium molybdate
6. Sodium hydrogen arsenate
7. Potassium sodium tartate
8. H_2SO_4

Chemical preparation

- Somogyi I: 180 g Na_2SO_4 (anhydrous) is dissolved in 600 ml of carbon dioxide free water. The solution was stirred until Na_2SO_4 (anhydrous) is dissolved completely. Then 15 g potassium sodium tartate is added. After solid chemical compounds dissolved completely, 30 g $NaCO_3$ and 20 g $NaHCO_3$ are added. The volume of the mixture solution is adjusted to 100 ml and kept for 1 day before used.
- Somogyi II: 72 g Na_2SO_4 (anhydrous) is dissolved in 300 ml of carbon dioxide free water. Then 8 g $CuSO_4.5H_2O$ is added. The volume of the mixture solution is adjusted to 400 ml and kept for 1 day before used.
- Working somogyi reagent: Working somogyi was obtained by mixing 4 parts of Somogyi II with 1 part of Somogyi I.
- Nelson reagent: 100 g Ammonium molybdate is dissolved in 1800 ml of carbon dioxide free water. 84 ml H_2SO_4 is added. Then 12 g sodium hydrogen arsenate

is added. The volume of the mixture solution is adjusted to 1000 ml and kept for 1 day before used.

Method

- 1 ml A suitable diluted sample is added into tube (duplicate is done).
- 1 ml Working somogyi reagent is added.
- Mixture solution is boiled for 15 min (red precipitant is obtained; Cu_2O). The reaction is stopped by put sample tubes in cool water.
- 1 ml Nelson reagent is added.
- 4 ml Distilled water is added.
- Absorbance at 520 nm is measured.
- The concentration of glucose is determined standard curve generated from different glucose concentration including 20, 40, 60, 80, 100 µg/ml.

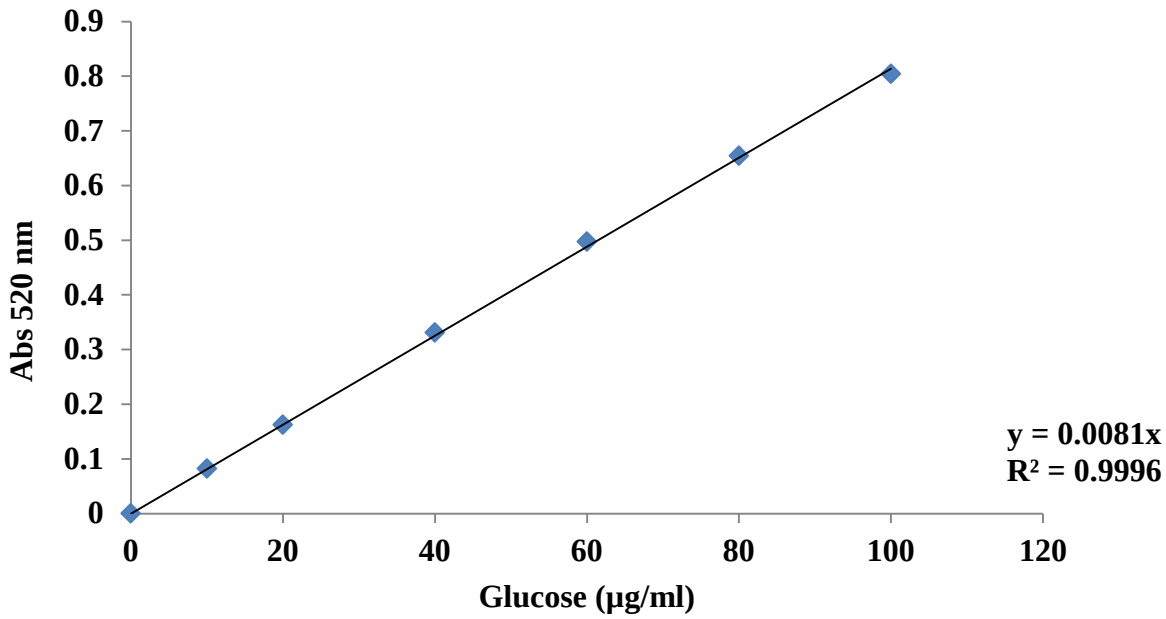


Figure 1 Standard curve of glucose generated by Nelson –Somogyi's method

The concentration in sample is determined according to equation (9);

$$y=0.0081x \quad (9)$$

When x is glucose concentration (µg/ml) and y is absorbance at 520 nm.

2. Protein Analysis by Biuret Method

Reagents

1. $CuSO_4$
2. Sodium potassium tartate
3. NaOH

Chemical preparation

- 1.5 g $CuSO_4$ and 6 g sodium potassium tartate were dissolved in 500 ml distilled water with constant stirrer.
- 300 ml 10 % NaOH was added.
- The volume of mixture solution was adjusted to 1000 ml by distilled water.
- The Biuret solution was kept in dark condition before use for 1 day.

Procedure

1ml Sample

⬇

4 ml Biuret solution

⬇

Stand allowed at room temperature for 30 min

⬇

Centrifugation

⬇

Absorbance at 570 nm

⬇

Comparing to standard curve

Standard curve preparation

No.	1	2	3	4	5
BSA 10 mg/ml	0	0.2	0.4	0.6	0.8
Distilled water (ml)	1	0.8	0.6	0.4	0.2
Biuret (ml)	4	4	4	4	4
Total volume (ml)	5	5	5	5	5

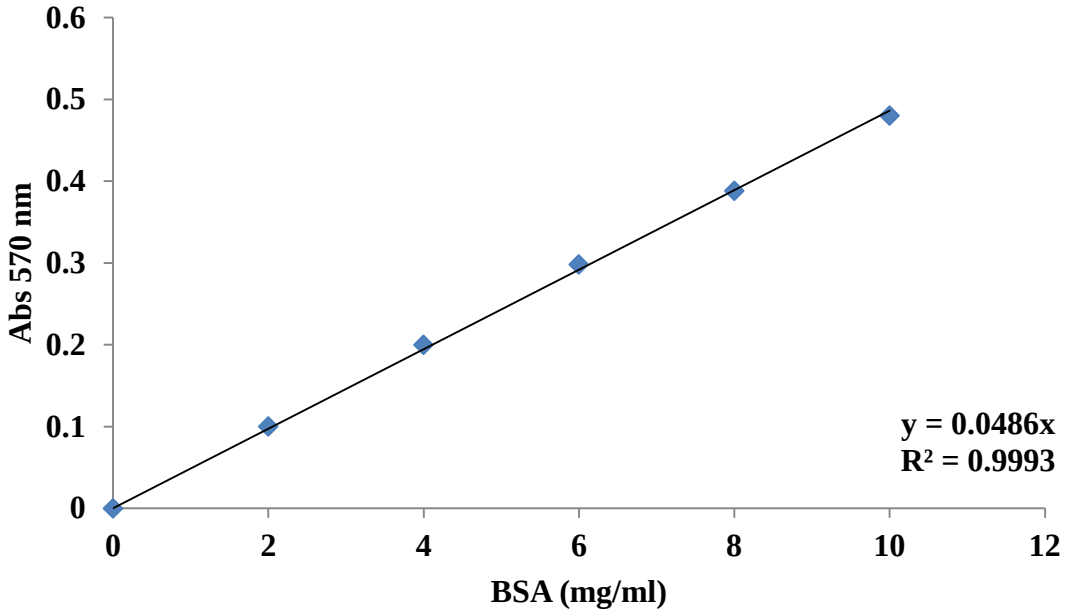


Figure 2 Standard curve of BSA generated by Biuret's method

The protein concentration in sample is determined according to equation (10);

$$y=0.0486x \quad (10)$$

When x is BSA concentration (mg/ml) and y is absorbance at 570 nm.

3. Lowry Method for Protein Content Determination

Reagents

A: 2% Na_2CO_3 in 0.1 N NaOH

B: 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate

C: 1 N phenol reagent

Method

1. Mix reagent A with reagennt B (50:1) Reagent (D)
   Use as soon as possible because it creates sediment after keeping long time
2. 3 ml Reagent D and 0.6 ml sample are added into test tube
3. Mixing and keeping and room temperature
4. 0.3 ml Reagent C is added and mixed immediately
5. Keeping at room temperature for 30 minute
6. Measuring at 750 nm
7. Calculating the protein content compared to standard curve

Standard curve of protein

1. By using BSA, protein solution of 30-300 µg/ml was prepared.
2. 3 ml Reagent D 0.6 ml BSA solution are added into test tube.
3. Following the process of upper part NO.3
4. Making the standard curve of protein

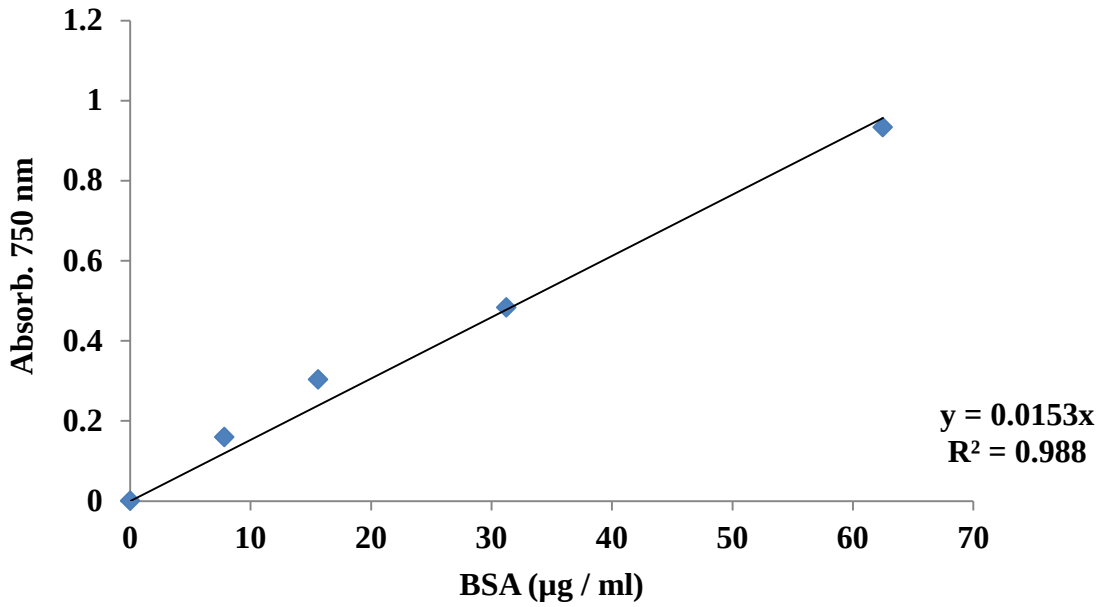


Figure 3 Standard curve of BSA generated by Lowry's method

The protein concentration in sample is determined according to equation (11);

$$y=0.1156x \quad (11)$$

When x is BSA concentration (µg/ml) and y is absorbance at 750 nm.

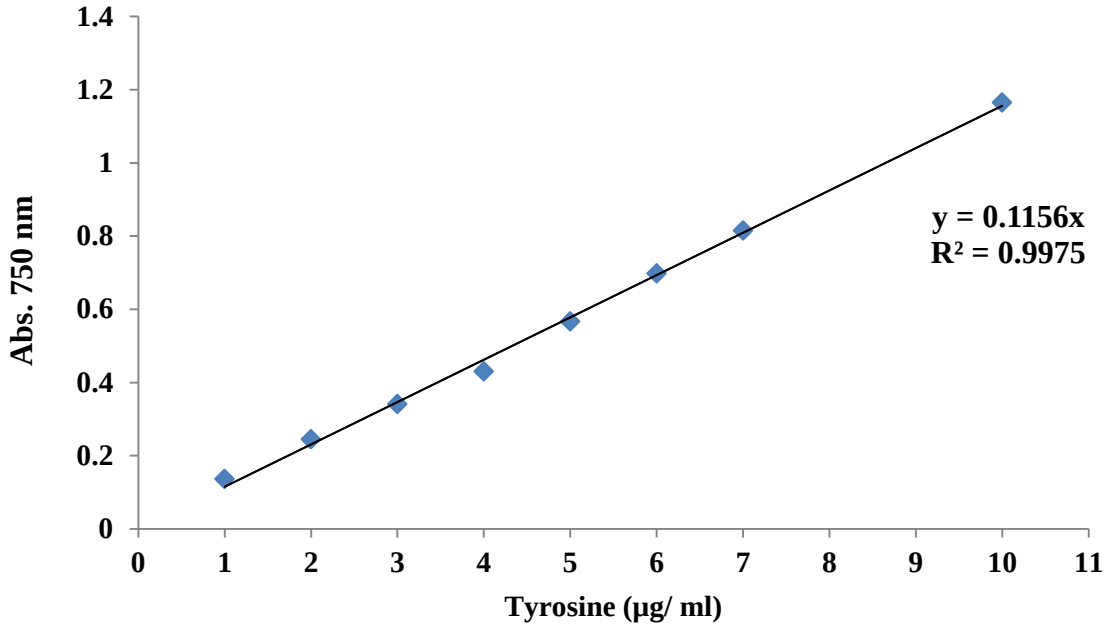


Figure 4 Standard curve of tyrosine generated by Lowry's method

The protein concentration in sample is determined according to equation (12);

$$y=0.1156x \quad (12)$$

When x is Tyrosine concentration (µg/ml) and y is absorbance at 750 nm.

3. Protein Determination by 280 nm

Reagents

1. Tyrosine (10 µg/ml as stock)
2. Distilled water

Method

1. Making various concentration of tyrosine at 0, 1, 2, 3, 4, 5, 10 µg/ml
2. Measuring at 280 nm

Table 1 Making standard for Tyrosine

Tyrosine (µg/ml)	**Absorb. 280 nm**
0	0
1	0.085
2	0.179
3	0.271
4	0.355
5	0.445
10	0.877

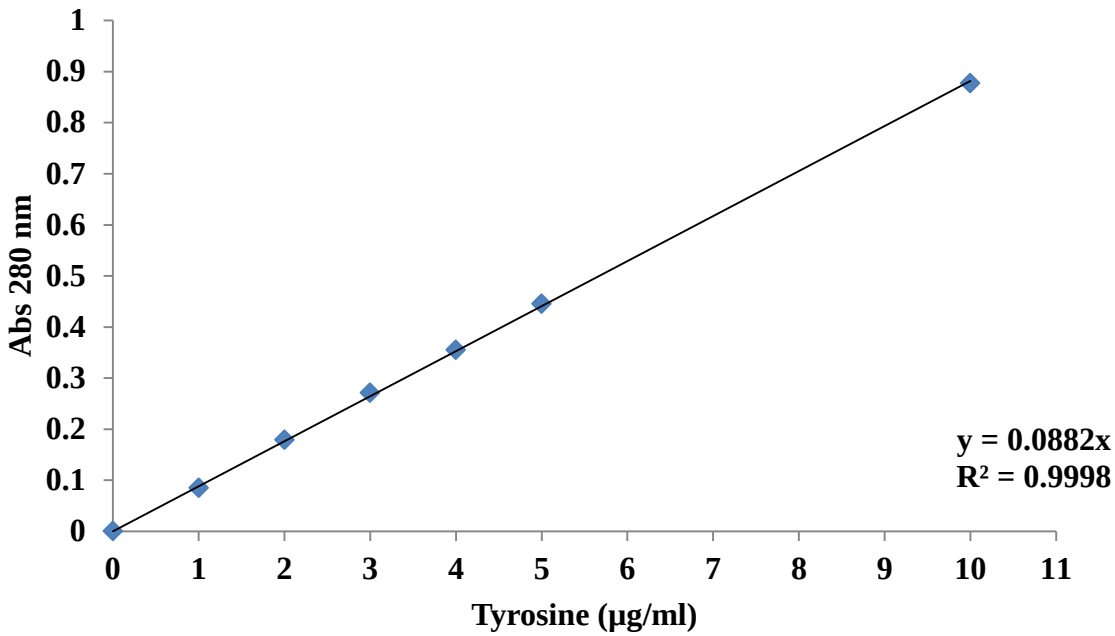


Figure 5 Standard curve of tyrosine generated by absorbance at 280 nm

The amino acid concentration in sample is determined according to equation (13);

$$y=0.0882x \quad (13)$$

When x is Tyrosine concentration (µg/ml) and y is absorbance at 280 nm.

4. N Acetyl Glucosamine

Reagents

1. N acetyl glucosamine
2. 0.85% H_3PO_4

Method

Standard solution of GlcNAc was prepared in 0.85% phosphoric acid at concentrations of 0, 12.8; 25.6; 38.4; 51.2 and 64.0 µg/ml. 0.85% H_3PO_4 was used as the reference liquid.

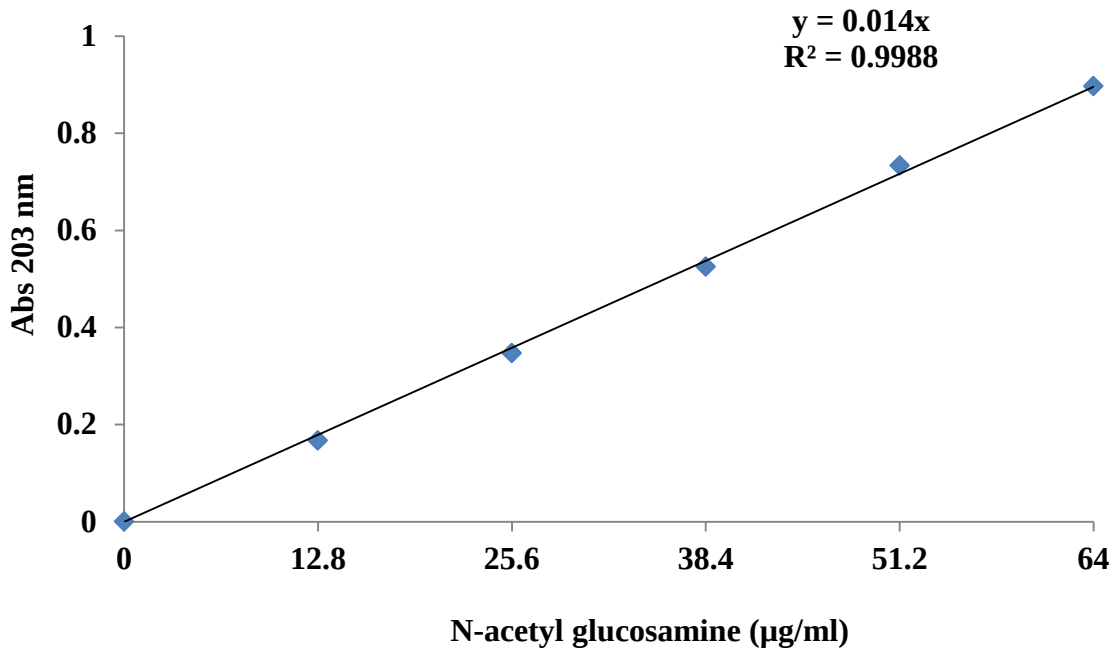


Figure 6 Standard curve of N acetyl glucosamine by absorbance at 203 nm

The N acetyl glucosamine concentration in sample is determined according to equation (14);

$$y=0.014x \quad (14)$$

When x is N acetyl glucosamine concentration (µg/ml) and y is absorbance at 203 nm.

APPENDIX C

ANALYSIS

1. Determination of Crude Protein in Shrimp Shells

Reagents

1. 0.5 M Sodium hydroxide
2. Biuret solution

Apparatus

1. Spectrophotometer
2. Glass cuvette
3. Test tube

Procedure

- 100 mg dried material was digested with 10 ml 0.5 M NaOH for 4 h at 40^{o}C.
- The mixture of alkaline solution was collected by centrifugation and used to estimate the protein concentration.
- The protein in alkaline solution was estimated by Biuret method (See in appendix B).
- Concentration of protein in sample was determined by comparing to standard curve generated form BSA (see in appendix B)**.**

2. Protease Assay by Using Azocasein as Substrate

Reagents

1. Azocasein **(**Sigma-Aldrich**)**
2. 50 mM Tris buffer pH 8.0
3. Trichloroacetic acid
4. Sterilized distilled water
5. 5 N HCl

Apparatus

1. Microplate reader
2. Microtiter plate

Reagent preparation

1. 50 mM Tris buffer pH 8.0 was prepared by dissolving 0.6 g Tris base with 500 ml sterilized distilled water and pH of solution was adjusted to pH 8 by 5 N HCl. Final volume was adjusted to 1000 ml
2. 0.5 g Azocasein **(**Sigma-Aldrich**)** was dissolved in 100 mM Tris buffer pH 8.0 and constantly stirred overnight at room temperature.
3. 10 g Trichloroacetic acid was dissolved by sterilized distilled water and final volume was adjusted to 100 ml

Procedure

- The supernatant (crude enzyme) was collected from the culture by centrifugation at 10,000 × g, at 4°C for 10 min.
- 120 µl of a suitable dilution of enzyme solution was added to 480 µl of azocasein (0.5 % in 50 mM Tris buffer pH 8.0 containing 5 mM $MgCl_2$) and the mixture was incubated at 37°C for 60 min.
- The reaction was stopped by adding 600 µl of 10% trichloroacetic acid, mixing, sitting for 30 min at 4^oC.
- The liquid was separated from the precipitate by centrifugation at 10,000 × g, at 4 °C for 15 min.
- 400 µl of 1.8 N NaOH was added into 800 µl of the reaction liquid, mixed and measured the absorbance at 420 nm (A_{420}) using a microplate reader (Varian Cary 50 MPR, Varian, Inc., USA).
- One unit of enzyme activity was defined as the amount which yielded an increase in A_{420} of 0.01 in 60 min at 37°C.

3. Protease Assay by Using Casein as Substrate

Reagents

1. Casein form milk **(**Sigma-Aldrich**)**
2. 50 mM Tris buffer pH 7.0
3. Trichloroacetic acid
4. 5 N HCl

Apparatus

1. Spectrophotometer
2. Glass cuvette

Reagent preparation

1. 50 mM Tris buffer pH 8.0 was prepared by dissolving 0.6 g Tris base with 500 ml sterilized distilled water and pH of solution was adjusted to pH 7 by 5 N HCl. Final volume was adjusted to 1000 ml
2. 1.0 g Casein **(**Sigma-Aldrich**)** was dissolved in 100 mM Tris buffer pH 7.0 and constantly stirred overnight at room temperature.
3. 20 g Trichloroacetic acid was dissolved by sterilized distilled water and final volume was adjusted to 100 ml

Procedure

- 0.5 ml of cell free supernatant, suitably diluted, was used as crude preparation to measure protease activity and was mixed with 0.5 ml 50 mM Tris –HCl (pH 7.0) containing 1% casein.
- The mixed solution was incubated at 50 ^{o}C for 20 minute.
- The reaction was stopped by addition of 0.5 ml TCA (20% w/v).
- The acid mixture was allowed to stand at room temperature for 15 minutes and centrifuged at 13,000 rpm for 15 minutes to remove the precipitate.
- The acid solution was estimated by using spectrophotometric measurement at 280 nm.
- The amount of free amino acid in acid mixture was determined by comparing to standard curve generated by tyrosine.

- A standard curve was generated by using solution of 0-0.5 mg/l of tyrosine (see in appendix B).
- One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under experimental assay condition.

4. Total Titratable Acid (TTA)

Reagents

1. Phenolphthalein
2. Free carbon dioxide distilled water
3. 0.1 N NaOH
4. Ethanol

Apparatus

1. 250 ml flask
2. Burette**e**

Chemical preparation

Phenolphthalein was dissolved in 95% ethanol to obtain 1% Phenolphthalein solution.

Procedure

- 4 ml of cell free supernatant was used as sample for determination of TTA
- Sample was added into 20 ml free carbon dioxide distilled water.
- Acid mixture was titrated by 0.1 N NaOH until pink color was obtained.
- The concentration of acid was determined in percentage according to equation (14);

$$TTA\ (\%) = (0.1 \times V \times 100 \times 90.08)/1000 \times 4 \quad (14)$$

When

V is volume of used titrant in ml

5. Caroteinoids Extraction

Reagents

1. Isopropyl alcohol
2. Hexane

Apparatus

1. 250 ml flask
2. Separatory funnel
3. Rotary evapurator

Chemical preparation

Mixture of isopropyl alcohol:n-hexane, 40:60 v/v

Procedure

- The carotenoid content in decalcified shrimp shell, reported as astaxanthin, was determined using the organic solvent extraction methodology
- 50 g decalcified shrimp shell was put into 250 ml mixture organic solvent to extract caroteinoid for 3 times.
- After solvent extraction, mixture organic solvent was removed by evaporator
- Astaxanthin concentration was recovered by addition of hexane to dissolve pigments.
- A suitable dilution was obtained by dilution of astaxanthin with haxen and concentration of astaxanthin in solution was determined by spectroscopic method at 472 nm.
- Hexane was used as the reference liquid.
- The amount of astaxanthin in decalcified shrimp shell was determined according to equation (15)

$$\text{Astaxanthin (µg/ml or mg/l)} = \text{Absorbance} \times \frac{\mathbf{10000}}{\mathbf{2100}} \qquad (15)$$

APPENDIX D

CULTURE MEDIA

1. Nutrient Broth

Peptone	5 g
Beef extract	3 g
Yeast extract	3 g
Sodium chloride	5 g
Distilled water	1000 ml

Final pH: 7.3 ± 0.2 at 25°C

Sterilized at 121 ^{o}C for 15 min

2. Nutrient Agar

Peptone	5 g
Beef extract	3 g
Yeast extract	3 g
NaCl	5 g
Agar	15 g
Distilled water	1000 ml

Final pH: 7.3 ± 0.2 at 25°C

Sterilized at 121 ^{o}C for 15 min

3. Tryptic Soy Broth

Enzymatic digest of casein	17 g
Enzymatic digest of soybean meal	3g
Sodium chloride	5 g
Dipotassium phosphate	2.5 g
Dextrose	2.5 g
Distilled water	1000 ml

Final pH: 7.3 ± 0.2 at 25°C
Sterilized at 121 ^{o}C for 15 min

4. Shrimp Shell Medium

Shrimp shell powder	20 g
Dipotassium phosphate	1g
Magnesium sulfate	0.5g
Distilled water	1000 ml

Final pH: 8.0 ± 0.2 at 25°C
Sterilized at 121 ^{o}C for 15 min

5. Gelatin Medium

Peptone	4 g
Yeast extract	1 g
Gelatin	15g
Distilled water	1000ml

Suspend ingredients with constant stirring to prevent scorching gelatin, and boil to dissolve gelatin. Adjust to pH 7.2 ± 0.2. Autoclave 15 min at 121°C.

6. Triple Sugar Iron Agar (TSI)

Polypeptone	20 g
Lactose	10 g
Sucrose	10 g
Glucose	1 g
Sodium Thiosulfate	2 g
Ferric Ammonium Sulfate	0.2 g
Sodium Chloride	5.0 g
Agar	13.0 g
Phenal Red	0.025 g.

Final pH ~ 7.3 ± 0.2

Suspend ingredients with constant stirring to prevent scorching gelatin, and boil to dissolve gelatin. Adjust to pH 7.2 ± 0.2. Autoclave for15 min at 121°C.

7. Simmons Citrate Agar

Sodium citrate	2 g
NaCl	5 g
K_2HPO_4	1 g
$NH_4H_2PO_4$	1 g
MgSO4	0.2 g
Bromthymol blue	0.08g
Agar	15 g
Distilled water	1000 ml

Heat gently with occasional agitation. Boil 1-2 min until agar dissolves. Fill 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclave 15 min at 121°C. Before medium solidifies, incline tubes to obtain 4-5 cm slants and 2-3 cm butts. Final pH, 6.8 ± 0.2.

8. Starch Agar

Peptone	5 g
Beef extract	3 g
Yeast extract	3 g
Potato starch	10g
Distilled water	1000 ml

Final pH: 7.3 ± 0.2 at 25°C

Heat to dissolve agar in 500 ml water. Dissolve starch in 250 ml water. Combine and dilute to 1 liter. Autoclave 15 min at 121°C.

9. Purple Carbohydrate Broth

Peptone	10 g

Carbohydrate	10 g
Beef extract	1 g
NaCl	5 g
Bromcresol purple	0.02 g
Distilled water	1000 ml

Dissolve either 5 g dulcitol, 10 g lactose, or 10 g sucrose in this basal broth
Dispense 2.5 ml portions into 13 x 100 mm test tubes containing inverted 6 x 50 mm fermentation tubes. Autoclave 10 min at 118 °C. Final pH, 7.4 ± 0.2.

10. MR-VP Medium

Peptone	7 g
Glucose	5 g
K_2HP0_4	5 g
Distilled water	1000 ml

Dissolve ingredients in water with gentle heat if necessary. Dispense 10 ml into 16 x 150 mm test tubes and autoclave 15 min at 118-121°C. Final pH, 6.9 ± 0.2.

- **Methyl Red Indicator**

Methyl red	0.1 g
Ethanol, 95%	300 ml

Dissolve methyl red in 300 ml ethanol. Bring volume to 500 ml with distilled water

- **Voges-Proskauer (VP) Test Reagents**

Solution 1

α-Naphthol	5 g
Alcohol	100 ml

Solution 2

Potassium hydroxide	40 g

Distilled water to make 100 ml

Voges-Proskauer (VP) Test

Transfer 1 ml of 48 h culture to test tube and add 0.6 ml solution 1 and 0.2 ml solution 2. Shake after adding each solution. To intensify and speed reaction, add a few creatine crystals to mixture. Let stand at room temperature. Read results 4 h after adding reagents.

11. Catalase Test

Pour 1 ml 3% hydrogen peroxide over growth on slant culture. Gas bubbles indicate positive test. Alternatively, emulsify colony in l drop 3% hydrogen peroxide on glass slide. Immediate bubbling is positive catalase test. If colony is taken from blood agar plate, any carry-over of red blood cells can give false-positive reaction.

12. SIM Motility Medium

Pancreatic digest of casein	20 g
beef extract	6 g
Ferrous ammonium sulfate	0.2 g
Sodium thiosulfate	0.2 g
Agar	3.5 g

Rehydrate and add 6 ml medium per 16 x 125 mm screw-cap tube. Sterilize at 121 oC for 15 min. Final pH, 7.3 ± 0.2.

13. MRS agar

Glucose	20 g
Peptone	10 g

Beef extract	5 g
Yeast extract	5 g
Sodium acetate	5 g
Triammonium citrate	2 g
K_2HPO_4	2 g
$MgSO_4.7H_2O$	0.1 g
$MnSO_4.4H_2O$	0.05 g,
tween 80	1 g
Agar	15 g
Distilled water	1000 ml

Autoclave 15 min at 121°C.

14. Bacillus cereus Selective Agar (Oxcoid)

Peptone	1g
Mannitol	10g
Sodium chloride	2 g
Magnesium sulphate	0.1 g
Disodium hydrogen phosphate	2.5g
Potassium dihydrogen phosphate	0.25g
Bromothymol blue	0.12g
Sodium pyruvate	10g
Agar	15g

pH 7.2 ± 0.2

Sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add the contents of one vial of Polymyxin B Supplement reconstituted as directed, then add 25ml of sterile Egg Yolk Emulsion. Mix well and pour into sterile Petri dishes.

15. Egg York Agar

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Cystine	0.4 g

Yeast Extract	5.0 g
Hemin	5.0 mg
Sodium Chloride	5.0 g
Egg Yolk Suspension	100.0 mL
Agar	20.0 g

Sterilise by autoclaving at 121°C for 15 minutes.

16. Nitrate Broth

Beef extract	3 g
Peptone	5g
KNO_3	1g
Distilled water	1000 ml

Dissolve ingredients. Dispense 5 ml portions into 16 x 125 mm tubes. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

17. YPD Medium

Yeast extract	10g
Peptone	20g
Glucose	20g
Distilled water	1000 ml

Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2. To obtain solid medium, 15 g agar is added.

APPENDIX E

CHEAP CULTURE MEDIUM for *Saccharomyces cerevisiae*

1. Medium preparation

- Fermenting liquid was collected by centrifugation at 10,000 rpm, 4^{o}C for 20min.
- pH of fermenting liquid was adjusted by 5 N HCl until pH of liquid was 6.6.
- Reducing sugar in fermenting liquid was determined according to appendix B.
- The reducing sugar in fermenting liquid was adjusted to 2% by glucose.

2. Inoculum preparation

- *Saccharomyces cerevisiae* obtained from department of microbiology, KMUTT was cultured in YPD medium for overnight at 37^{o}C and 200 rpm.
- Cell pallet was collected by centrifugation at 10,000 rpm, 4^{o}C for 20 min and washed with 0.85% NaCl for twice.
- Cell density was adjusted to 0.6 at absorbance of 600 nm (OD_{600}) used as starter.

3. *Saccharomyces cerevisiae* growth in medium having fermenting liquid as a major component

- 0.1 % starter was added into YPD medium, fermenting liquid medium.
- All cultures were incubated at 37^{o}C, 200 rpm for 3 days.
- Cell concentration was determined by spread plate technique.

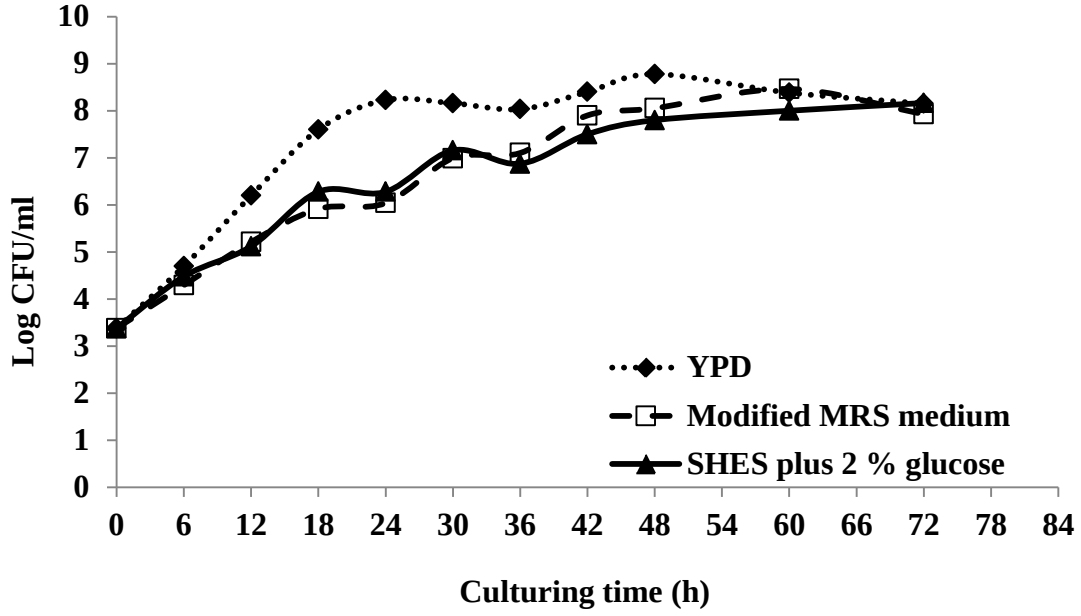


Figure 7 Culturing *Saccharomyces cerevisiae* in YPD medium and fermenting liquid obtained from different inoculum mediums

From the Figure 7, the result showed that *Saccharomyces cerevisiae* could grow well in YPD medium which cell concentration reached maximum at 8.77 Log CFU/ml after 48 h. Medium made from fermenting liquid obtained from different inoculum mediums including modified MRS medium and SHES plus 2% glucose could also support the growth of *Saccharomyces cerevisiae*, but maximal cell concentration reached 8.0 Log CFU/ml at 60 h after incubation.

APPENDIX F

SDS-PAGE (POLY ACRYLAMIDE GEL ELECTROPHORESIS)

Running buffer (10X) for protein SDS-PAGE

Tris –base (30g), glycine (144g) and 10% SDS (100ml) and final volume as 1 liter, (Working volume is 1X)

Coomassie blue staining solution

50% (v/v) methanol

10 % (v/v) acetic acid

0.25% (w/v) Coomassie Blue R-250

Destaining solution

5 % (v/v) acetic acid

10% (v/v) methanol

Loading 2X buffer

250mM Tris-HCL, pH 6.8

2% SDS

10% glycerol

20mM DTT

0.01% bromophenol blue

PAGE (30%)

Acrylamide	29.2g
N,N'-methylene bis –acrylamide	0.8g
H_2O	100ml

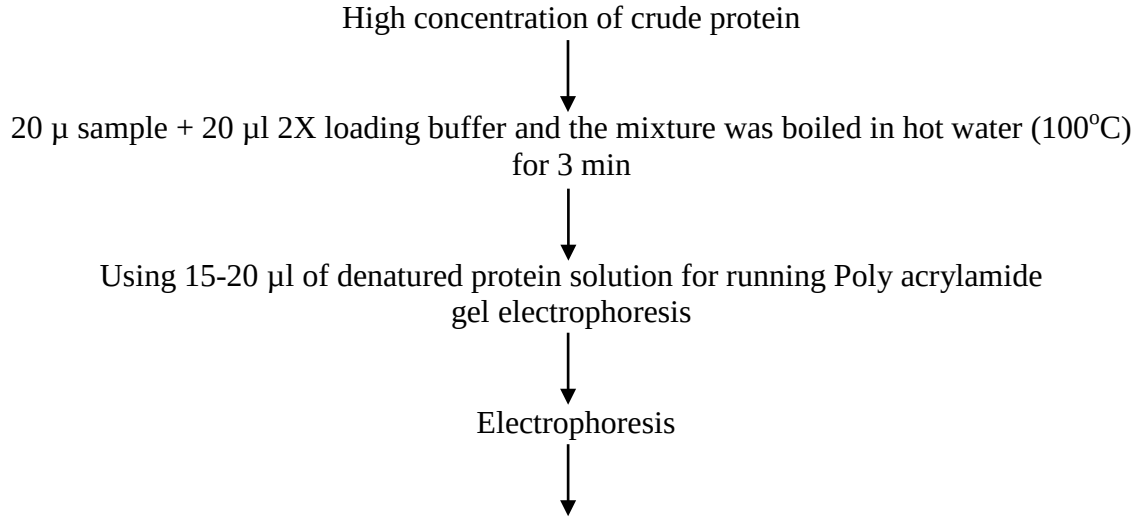
Resolving gel 5 ml (Acrylamide 12%)

1.5M Tris-HCl,pH8.8	1.3	ml
Water	1.6	ml
Acrylamide solution (30%)	2.0	ml
Ammonium persulphate(10%)	50.0	µl
SDS(10%)	50.0	µl
Total	5.0	ml
TEMED	5.0	µl

Stacking gel 1.9 ml (4%)

1.5M Tris-HCl, pH6.8	0.25	ml
Water	1.40	ml
Acrylamide solution (30%)	0.25	ml
Ammonium persulphate (10%)	20	µl
SDS (10%)	20	µl
Total	1.92	ml
TEMED	2	µl

Preparation of crude protein



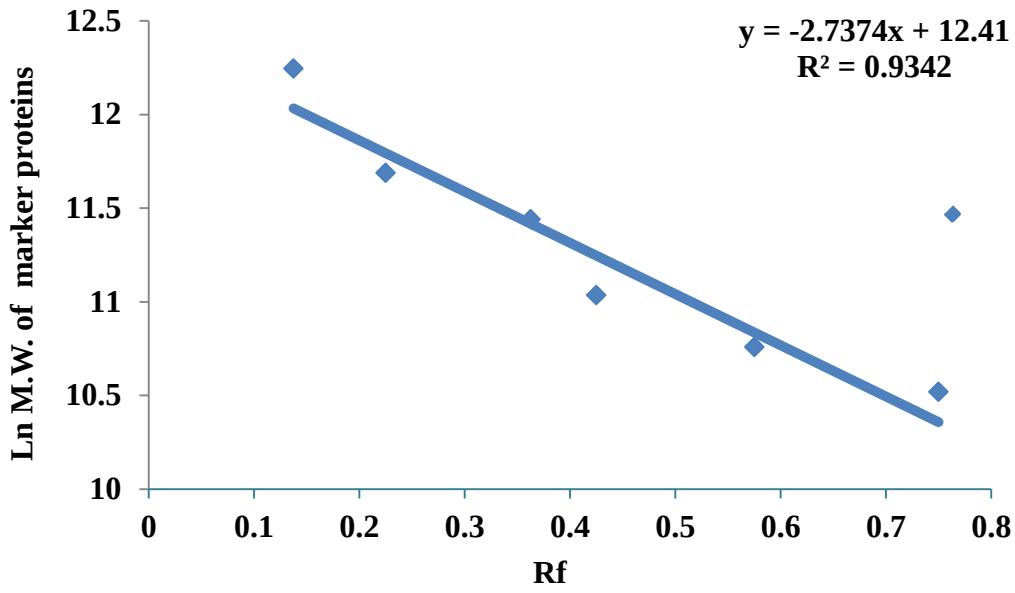


Figure 8 Linear model for determination of protein size

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