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Master's Degree Thesis

β-glucanase from *Bacillus* sp. CSB34: Screening, Production, Partial Purification, and Biochemical Characterization

Graduate School of Chosun University

College of Pharmacy

Young Kyun Kim

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Advisor: Prof. Jin Cheol Yoo, PhD

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ABSTRACT

β-glucanase from *Bacillus* sp. CSB34: Screening, Production, Partial Purification, and Biochemical Characterization

Young Kyun Kim

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Department of Pharmacy

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Cellulose, the most available sources of alkaline, is produced as a result of agricultural, industrial and forestry products. Because of its huge availability and renewability, it is considered a candidate for energy sources and feedstock. The increasing concern about fossil fuel shortages forces many researchers to work on to find new possible substances that can be applicable for the production of bio-ethanol from cellulosic materials. We carried out our experiment with the objective of exploring a potent new microbial source with efficient characteristics, which could be used as a weapon to make cellulose a waste free environment. We screened for bacterial strains capable of cellulose utilization, high enzyme production and biochemical characterization that could also be beneficial for biotechnological applications.

β-glucanase from *Bacillus sp*. CSB34 was isolated from popular traditional Korean food and successfully produced in carboxy methyl cellulose. It was purified to homogeneity from culture supernatant using ammonium sulfate precipitation, membrane concentration, dialysis, and followed by a two-step chromatographic separation by Sepharose CL-6B and Sephadex G-50. The molecular mass of the enzyme was approximately 50 kDa via 12.5 % SDS-PAGE and CMCase activity. The purified enzyme (GlucanaseCSB34) exhibited the optimal activity at pH 7.5 and 50 °C.

국문초록

Bacillus sp. CSB34 균주 유래 β-glucanase 의 생화학적 특성 연구

김용균

지도교수: 유진철

조선대학교 약학대학 약학과

셀룰로오스는 가장 흔한 알칼리성 물질임과 동시에 농업이나 산업, 그리고 임업을 통하여 주로 생산되고 있다. 셀룰로오스는 많은 양의 생산량을 확보할 수 있고 높은 재생가능성 때문에, 에너지원과 주된 공급 원료로 각광받고 있다. 최근 들어 화석 연료 부족에 대한 우려가 늘어나면서, 많은 전문가들은 셀룰로오스에서 추출하는 바이오에탄올 생산에 적용 가능한 새로운 물질을 찾기 위해 노력하고 있는 상황이다. 이러한 노력의 일환으로 잠재력 있고 효율적인 분해효소를 생산하는 미생물 균주를 찾는 것에 목적을 두고 실험을 진행하였으며, 이는 셀룰로오스 폐기물이 없는 환경을 만드는데 도움이 될 것으로 예상하고 있다. 따라서 본 실험에서는 섬유소 활용과 높은 효소 생산이 가능하면서도 우수한 생화학적 특성을 바탕으로, 다양한 생명공학산업 분야에 적용이 용이한 미생물균주의 분리 및 검사를 실시하였다.

한국의 고유 전통음식에서 분리한 *Bacillus* sp. CSB34 의 β-glucanase 는 carboxy methyl cellulose 에서 매우 효율적으로 생성되었다. *Bacillus* sp. CSB34 의 배양액은

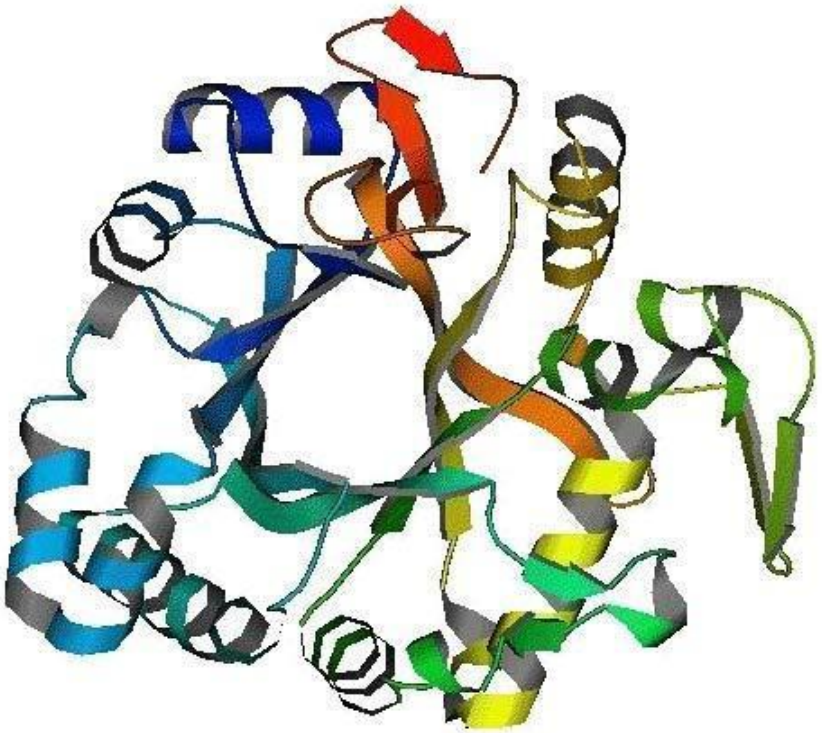
ammonium sulfate 를 이용한 침전법, 필터를 통한 농축 및 정제를 진행하였으며, Sepharose CL-6B 및 Sephadex G-50 을 사용하여 컬럼크로마토그래피를 통한 정제를 실시하였다. 효소의 분자량을 측정하기 위하여 CMCase 및 12.5% SDS-PAGE 전기영동을 통하여 단백질 밴드를 확인하였으며 그 결과 50kDa 으로 확인되었다. 정제된 효소인 GlucanaseCSB34 는 pH 7.5 와 50°C 에서 최적의 활성상태를 보였다.

ABBREVIATIONS

GRAS	Generally Regarded As Safe
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
RPM	Rotation per minute
kDa	Kilo Dalton
SDS-PAGE	Sodium Dodecyl Sulfate –Polyacrylamide Gel Electrophoresis
NCBI	National Centre for Biotechnology Information

CHAPTER 1: INTRODUCTION

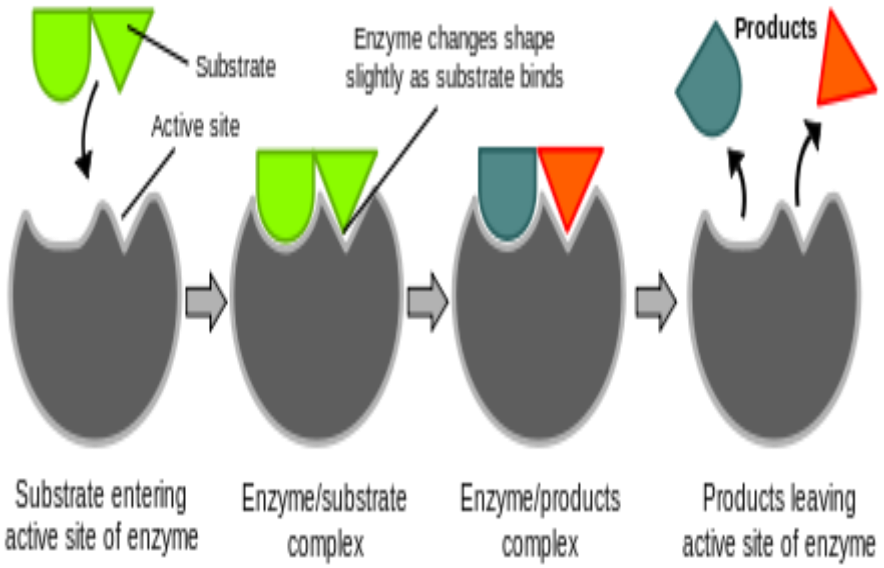
Enzymes are macromolecular biological catalysts that accelerate, or catalyse, chemical reactions. The component at the starting of the action are called substrates and the enzyme converts these components into different molecules known as products. These bio-catalysts for the biochemical interaction result in microbial growth, respiration, and the formation of fermentation products.



There are two types of enzymes:

ADAPTIVE: Produced only when the need arises such as when a cell is deficient of a particular nutrient.

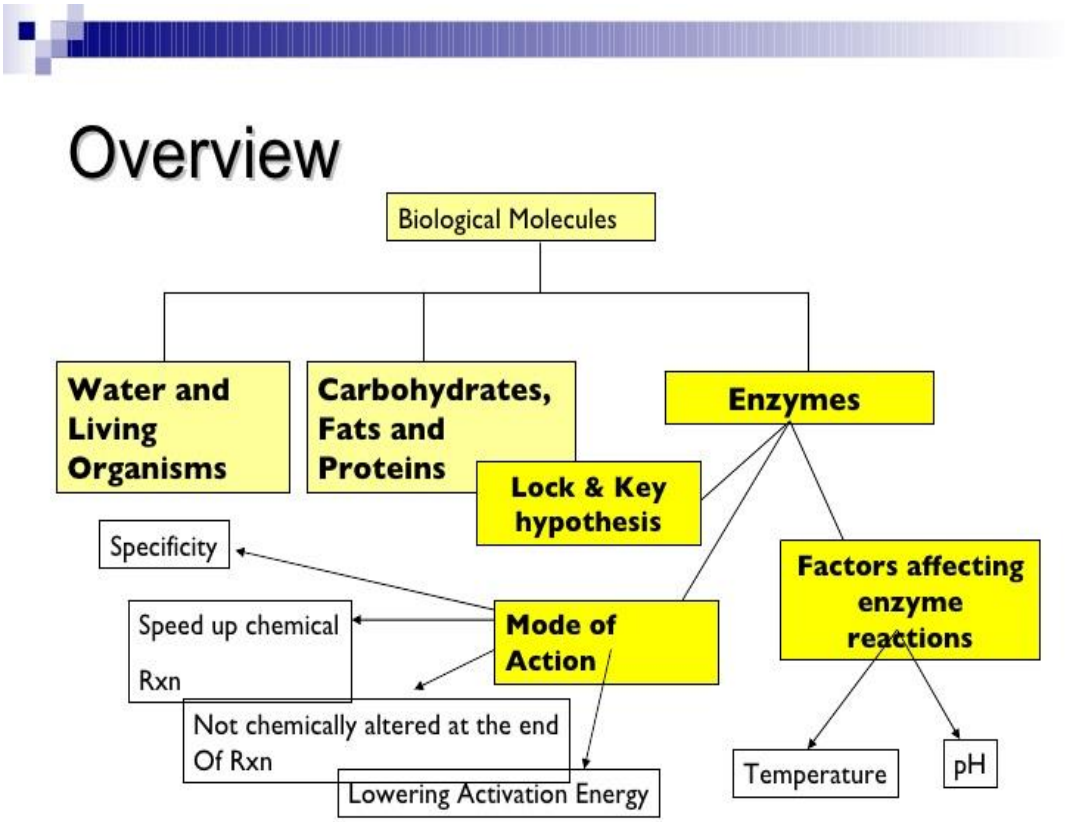
CONSTITUTIVE: Always produced irrespective of the amount of substrate.



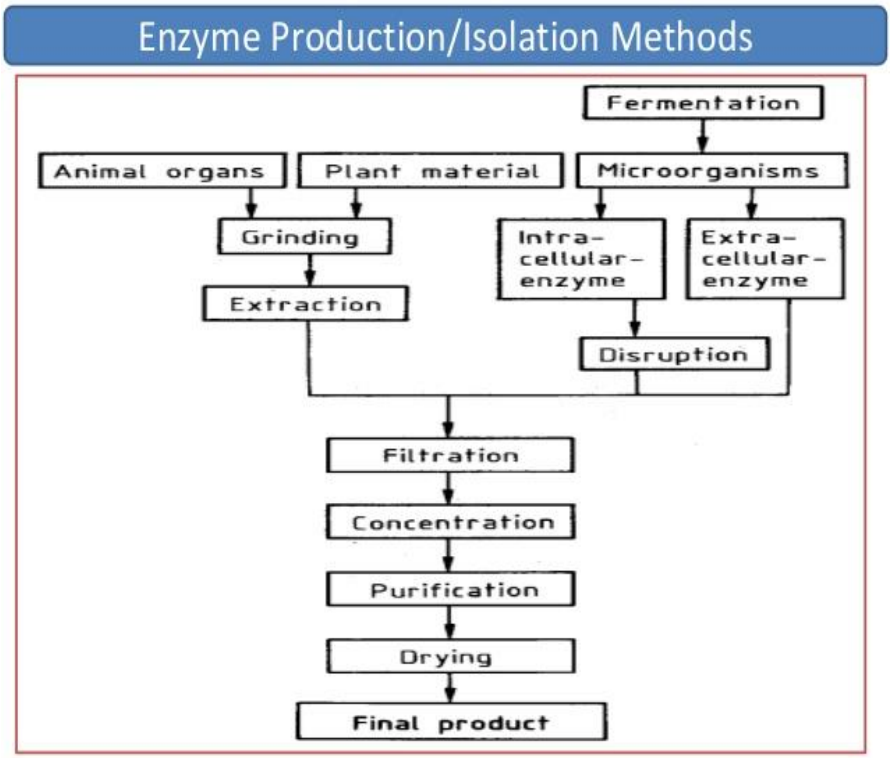
The first enzyme produced industrially was the fungal amylase Takadiastase which was employed as a pharmaceutical agent for digestive disorders. By 1969, 80 % of all laundry detergents contained enzymes, chiefly Proteases. Due to the occurrence of allergies among the production workers and consumers, the sale of enzyme-utilizing detergents decreased drastically.

Special techniques like micro-encapsulation of these enzymes were developed which could provide dustless protease preparation. It was thus made risk free for production workers and consumers. Microbial rennin is also one of the most significant enzymes. It has been used instead of Calf's rennin in cheese production

Location of Enzymes: Enzymes which are produced within the cell or at the cytoplasmic membrane are called Endocellular enzymes. Enzymes which are liberated in the fermentation medium which can attack large polymeric substances are termed as Exocellular enzymes. Such as; Amylases, Proteases and Glucanase.



Prospects of Enzyme Application: Microbial Genetics – High yields can be obtained by Genetic manipulation. For eg.– genetically, *Hansenula polymorpha* has been modified so that 35% of its total protein holds the enzyme alcohol oxidase. Optimization of fermentation conditions (Use of low cost nutrients, optimal utilization of components in nutrient solution, temperature and pH). New cell breaking methods like Homogenizer, Bead mill, Sonication etc. Modern purification processes like Counter current distribution, Ion-exchange chromatography, Molecular-sieve chromatography, Affinity chromatography and precipitation by using alcohol, acetone. Immobilization of enzymes. Continuous enzyme production in special reactors.



Methods of Enzyme Production: Semisolid Culture and Submerged Culture.

Semisolid Culture: Enzyme culture is produced on the surface of a suitable semi-solid substrate (Moistened Wheat or Rice Bran with nutrients). Preparation of Production Medium – Bran is mixed with solution containing nutrient salts. pH is maintained at a neutral level. Culture medium is sterilized in an autoclave. After that medium is spread on metal container adjusted to a depth of 1-10 cm. The culture is transferred either after cooling in the autoclave or in trays. High enzyme concentration in a crude fermented material.

Enzymes produced by semi-solid culture enzyme micro-organisms α- Amylase *Aspergillus oryzae* Glucoamylase *Rhizopus* spp. Lactase *Aspergillus oryzae* Pectinase *Aspergillus niger* Protease *Aspergillus Niger & Aspergillus oryzae* Rennet *Mucor pusillus*.

Advantages of Semi-solid culture: It involves comparatively low investment and allows for the use of a substrate with high dry matter content. Hence it yields a high enzyme concentration in the crude fermented material. To cultivate those moulds which cannot grow in the fermenters due to wall growth. Allows the moulds to develop into their natural state.

Disadvantages of Semi-solid culture: Requires more space and more labour, involves greater risk of infection and it is difficult to introduce automation in such systems.

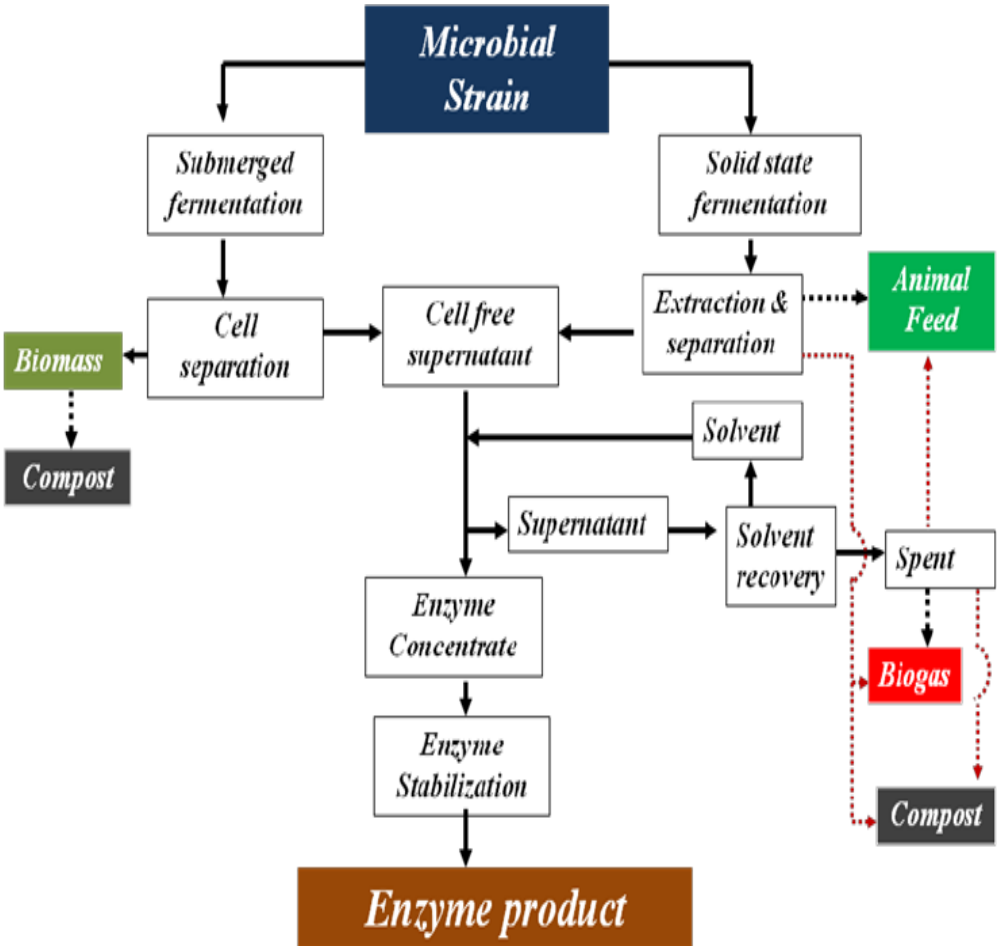
Submerged Culture: Fermentation equipment used is the same as in the manufacture of antibiotics. It’s a cylindrical tank of stainless steel and it is assembled 2along with an agitator, an air bubbling device with a cooling system and different ancillary equipment (Foam control, pH monitoring device, temperature, oxygen tension etc). Good growth is not enough to obtain a higher enzyme yield.

Presence of inhibitors or inducers should also be checked in the medium. Example – Presence of Lactose induces the production of β- galactosidase. As the inducers are expensive, constitutive mutants are used which do not require an inducer. Glucose represses the formation of some enzymes (α-amylases). Thus the glucose concentration is kept low. Either the glucose can be supplied in an incremental manner or a slow metabolizable sugar (Lactose or metabolized starch). Certain surfactants in the production medium increases the yield of certain enzymes. Non- ionic detergents (eg. Tween 80, Triton) are frequently used.

Advantages of Submerged culture: Requires less labor and space, low risk of infection, and automation is easier.

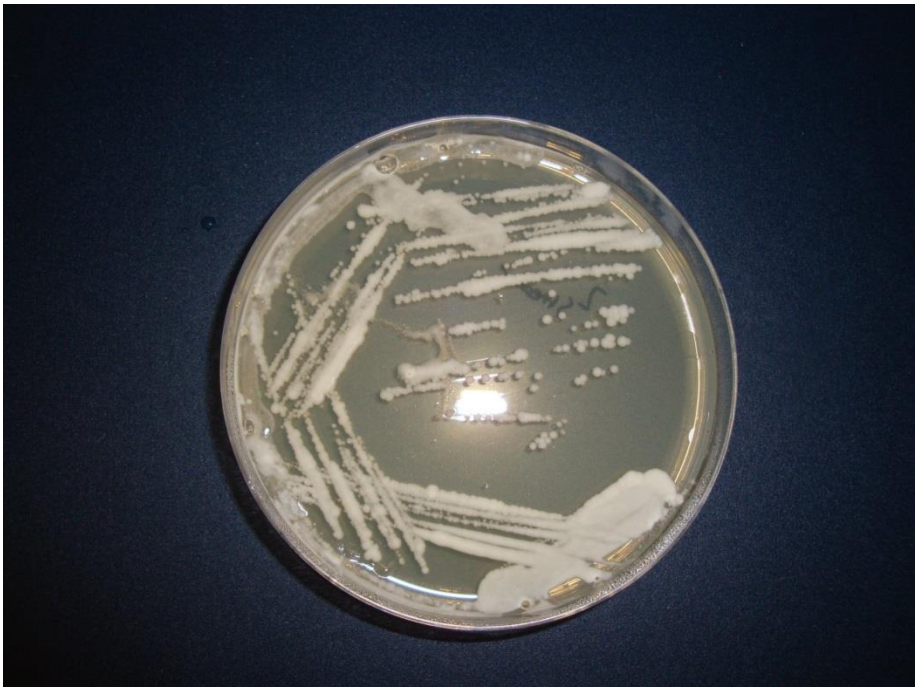
Disadvantage of Submerged Culture: Initial investment cost is very high.

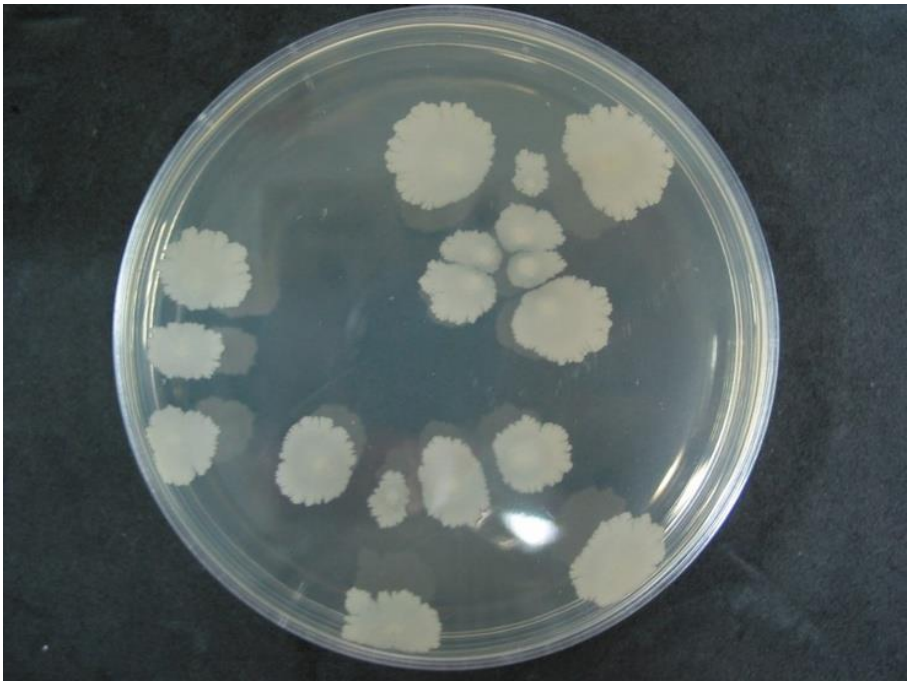
After fermentation: Once fermentation is finished, the final liquor obtained from it is subjected to fast cooling to 5 ^{o}C in order to reduce deterioration. Separation of micro-organisms is accomplished either by filtration or by centrifugation of the refrigerated broth with adjusted pH. To obtain a higher purity of the enzyme, it is precipitated with acetone, alcohols or inorganic salts (ammonium or sodium sulfate). In case of large scale operations, salts are preferred to solvents because of explosion hazards.



***Bacillus* sp.**

Bacteria are mostly primitive in form and shows one of three basic structures and *Bacillus* is one of them. The other two are coccus and spirillus [1]. Structurally, *Bacillus* species gram positive rod-shaped, endospore-forming as well as obligate aerobes or facultative anaerobes. In some cases, Gram-positive bacteria in cultures may turn Gram-negative with aging. Many species of the *Bacillus* genus, which is a member of the phylum Firmicutes, have a wide range of physiologic abilities which allow them to survive in most environmental conditions [2].

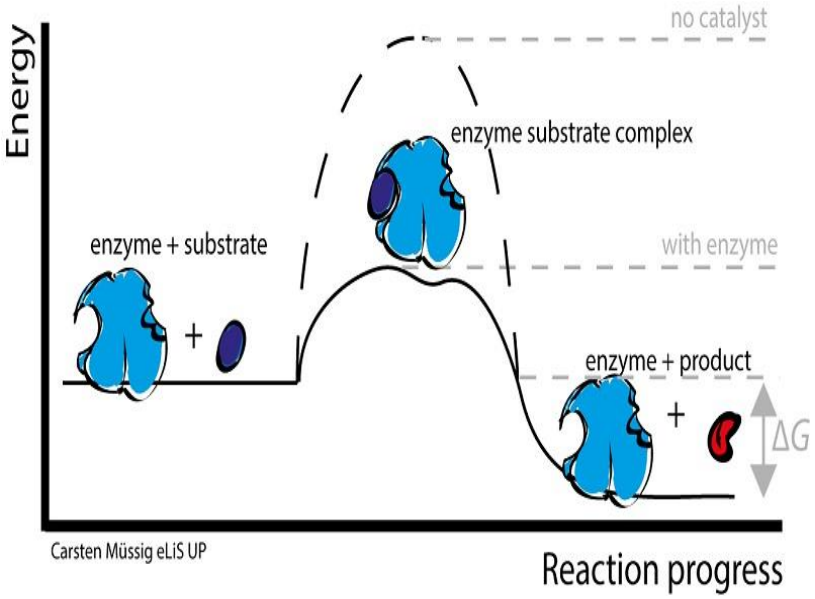


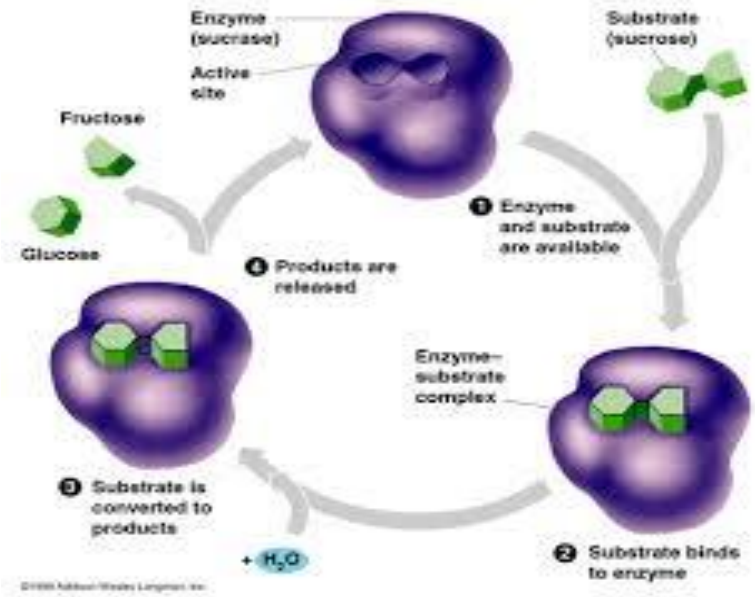


Bacillus species are bacterial workhorses in microbial fermentations. They are listed under the Food and Drug Administration's GRAS list. The ability of some *Bacillus* strains to produce and secrete large number of enzymes have kept them among the most important and essential industrial enzyme producers and are consider as the future bio-industrial catalysts. Various species are capable of fermentation in the acidic, neutral, and also alkaline pH ranges, including with the role of thermophiles in the particular genus, has summoned to the establishment of a different variety of new commercial available bio-industrial enzyme products with optimum temperature, pH, and various stability properties to cover a variety of specific applications [3]. *Bacillus* comprises a highly versatile and remarkable model microorganism for the research of generic stress and pathophysiology [4].

1.1 Microbial Enzyme

Enzymes are biocatalysts that play an important role in metabolic and biochemical reactions [5].Microorganisms are the primary source of enzymes, because they are cultured in large quantities in a short span of time and genetic manipulations can be done on bacterial cells to enhance the enzyme production [6–8]. In addition, the microbial enzymes have been given more attention due to their active and stable nature than enzymes from plants and animals [6–8]. Most of the microorganisms are unable to grow and produce enzyme under harsh environments that cause toxicity to microorganisms. However, some microorganisms have undergone various adaptations enabling them to grow and produce enzymes under harsh conditions [9, 10]. Recently several lines of study have been initiated to isolate new bacterial and fungal strains from harsh environments such as extreme pH, temperature, salinity, heavy metal, and organic solvent for the production of different enzymes having the properties to yield more [10–13].





1.2 Cellulase

Cellulose is a carbohydrate polymer and a main component of the cell wall of plants. It predominantly occurs in long water-insoluble, crystalline microfibrils. These microfibrils each consists of several parallel oriented linear chains of (1→4) β-linked D-glucose and are highly resistant to enzymatic hydrolysis.

β-endoglucanase activities are determined by the extent the natural polysaccharide substrate is hydrolyzed. Microorganisms can be screened for β-endoglucanase production by measuring their ability to grow on defined or semi-defined media by growing cultures in conjunction with an indicator of enzyme activity or by measuring the extracellular enzyme activity in spent fungal cultures. Growing cultures on specific media capable of enzyme detection is the simplest screening method, but it may take days to visualize enzyme activity. Alternatively, measuring cell-free enzyme activity in spent culture filtrates is more rapid but it may also require more processing.

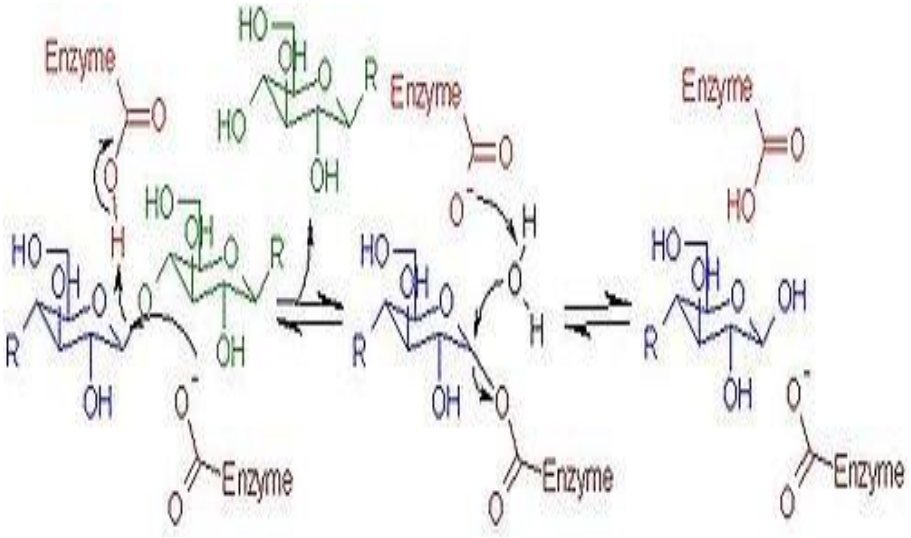
One of the most widely used methods to measure β-endoglucanase activity in liquid samples is the reducing sugar assay. The reducing sugar assay measures the amount of reduced sugars, such as glucose, present after a standard incubation of substrate with an enzyme sample. The reducing sugar assay can be adapted to a microplate format; however, its application is limited to samples with low reducing sugar levels. Samples already high in reducing sugars, such as spent culture filtrates grown in potato dextrose broth, or the use of poor quality assay substrate will result in spectrophotometric background values too high for analysis.

To exploit the energy available in cellulose, microorganisms produce a variety of mixtures of synergy acting cellulases [14]. Cellulases possess a catalytic domain which is linked by a cellulose binding domain to a glycosylated, Pro/ Thr / Serrich peptide [15]. The cellulose binding domain determines the efficiency of degradation of insoluble cellulose [16].

There are two classes of the cellulase systems. Non-complexed cellulase systems consist of a set of three soluble enzymes. The Endocellulase breaks down internal bonds to dismantle the fine crystalline structure of cellulose which ultimately produces short polysaccharide chains and small amounts of glucose monomers. The Exocellulase cuts 2 to 4 units from the end part of the exposed chains formed by the endo-cellulase, resulting in disaccharides such as cellobiose. Cellobiase or beta-glucosidase hydrolyses the β-glycosidic linkage between the two glucose molecules in the cellobiose leading to its constituent glucose monomers. Noncomplexed cellulase systems are typical of cellulose-degrading aerobic fungi.

Bacterial β-1, 3 and 1, 4-glucanases (EC 3.2.1.73) are members of the glycosyl hydrolase family 16 and specifically break down the β-1, 4-glycosidic joining to 3-O-substituted glucopyranose residues. The enzymatic de-polymerization of β-glucan releases mostly tri and tetra-saccharides, and the enzyme does not have a detectable effect on β-1, 4-d-glucan, CMC or on β-1, 3-d-glucans. β-1, 3 and 1, 4-Glucanases have potential in several industrial applications. Exogenous β-1,3 and 1,4-glucanases have been used to reduce the viscosity of complex barley β-glucans during the mashing process in the brewing industry and can also improve the β-glucan digestibility in poultry and swine feed stuffs. β-Glucans are abundant cell wall constituents of sugarcane, and the inclusion of β-1,3 and1,4-glucanases in hydrolase has been thought to

improve the formation of fermentable sugars via lignocellulosic feedstocks specially in biofuel production.



Complexed cellulase systems are composed of the same three enzyme types, but they are bound together forming a multi-protein complex. Complexed cellulases are typically found in anaerobic cellulose degrading bacteria.

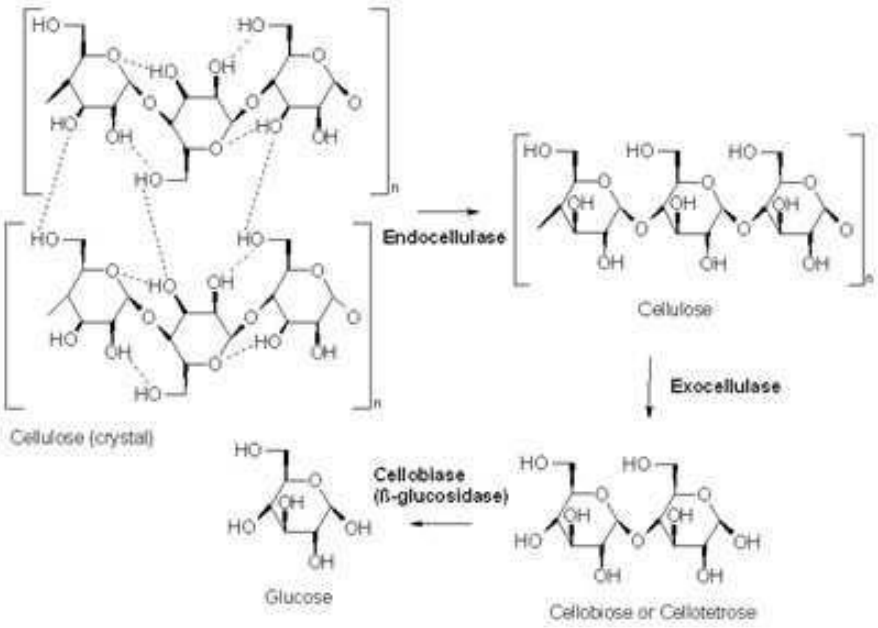


Figure 1.1: The three types of reaction catalyzed by cellulases: 1. endo-cellulase: breakage of the noncovalent interactions present in the crystalline structure of cellulose.

2. exo-cellulase: hydrolysis of the individual cellulose fibers to break it into smaller sugars.

3. Cellobiase: hydrolysis of disaccharides into glucose monomers.

(Figure from en.wikipedia.org/wiki/Image:Types_of_Cellulase2.png; Dec. 2008)

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Sepharose-Cl-6B and Sephadex G-50 were purchased from Pharmacia (Uppsala, Sweden). All the other reagents were of highest analytical grades.

2.2 Isolation and screening of β-glucanase

Seventy-eight samples of traditional Korean food (kimchi) collected from different provinces in Korea were selected for our study. Nature uses microorganisms to carry out the fermentation process, and for many years, humankind has used different microorganisms to make food products. The biological association of these microorganisms in humankind, their symbiotic relationship to environment and human health, led us to select food items rather than anything else for this study. For bacterial isolation, briefly, 1 g of kimchi was mixed with 0.9ml of 0.85% NaCl and incubated for 24 h at 37 °C. Serial dilutions were performed up to 10^{-7} in Mueller-Hilton broth. From each dilution, streaking was done to find out the appropriate colony-forming unit (CFU). The appropriately diluted solution was stored as stock cultures in 20% glycerol at −70 °C. All 78 bacterial strains were subjected to the screening process. The preliminary screening procedure was carried out by streaking the stock cultures on agar plates containing 1.25% (w/v) carboxy methyl cellulose as a carbon source, 0.5% (w/v) yeast extract and 0.5% (w/v) beef extract as nitrogen sources, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% K_2HPO_4, 0.07% KH_2PO_4,

and 0.05% NaCl as metal ion sources and 1.5% agar and incubating the plates at 37 °C for 30 h. Fully grown culture plates were flooded with 0.5% Congo red for 20 min and washed with distilled water. Thereafter, the plates were flooded with 1 M NaCl for 15–20 min and washed with distilled water 2–3 times. Results were interpreted on the basis of LBG utilization. Further screening was done by culturing in a media containing 1.25% (w/v) CMC, 0.5% (w/v) yeast extract, 0.5% (w/v) beef extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% K_2HPO_4, 0.07% KH_2PO_4, and 0.05% NaCl. The strains were cultured in 250 mL Erlenmeyer flasks containing 50 mL media at 37 °C with shaking at 120 rpm for 60 h. The culture broths were centrifuged at 10,000*g* for 30 min, and the supernatant was collected for enzyme activity measurements. Bacterial strain CSB34 exhibited the highest glucanase activity among the strains subjected to screening, as per agar plate assay and enzyme assay, and thus was selected for further study. Strain identification based on morphological characteristics was carried out according to Bergey's Manual of Systematic Bacteriology and further by 16S rRNA gene sequence analysis.

2.3 DNA sequence similarities

The BLAST program (www.ncbi.nlm.nih.gov/blast) was employed in order to assess the degree of DNA similarity.

2.4 Enzyme production and purification

Bacillus sp. CSB34 was cultured in 2 L baffled flasks containing 400 mL of CMC medium at 37 °C with shaking at 120 rpm for 60 h. 1 % (v/v) seed culture was inoculated into the CMC

medium grown on 0.4% (w/v) beef extract, 0.4% (w/v) peptone, 0.1% (w/v) yeast extract, and 0.25% (w/v) NaCl at 37 ℃ for 16 h. After 60 h of cultivation, the cell-free supernatants were collected by centrifugation at 10,000*g* for 30 min. The extracellular enzyme was then further purified in series of steps. All the purification processes were carried out at 0 ℃ unless stated otherwise. The supernatant was subjected to ammonium sulfate precipitation at 30–80% saturation overnight. The precipitate was collected by centrifugation at 10,000*g* for 50 min, re-suspended and dialyzed in 10 mM Tris/HCl (pH 7.5) buffer overnight, and concentrated using a 50 kDa ultrafiltration centrifugal device (Millipore Corp, Darmstadt, Germany). The enzyme solution was then loaded onto a Sepharose CL-6B column (85 cm × 1.7 cm) pre-equilibrated with 10 mM Tris/HCl, pH 7.5. Proteins were eluted at 30 mL/h and 3 mL fractions were collected. The glucanase active fractions were pooled, concentrated, and loaded on to a Sephadex G-50 column pre-equilibrated with 10 mM Tris/HCl, pH 7.5. Active fractions were pooled, concentrated, and analyzed for purity. Further characterizations were carried out using the pure enzyme.

2.5 Protein estimation and enzyme activity

The protein concentrations were determined at 595 nm according to the Bradford method [17] using bovine serum albumin as a standard.

Glucanase activities were assayed according to the method of Miller [18] and determined at 50 ℃ using CMC as a substrate. The reaction mixture containing 0.1 mL of 0.5% substrate and 0.1 mL of the suitably-diluted enzyme solution (10 mM, Tris/HCl, pH 7.5) was incubated for

30 min. Along with the reaction mixture, a control solution was assayed at 0 °C. The 3,5-dinitrosalicylic acid (DNS) method was employed to measure the released reducing sugar at 540 nm. A glucose standard curve was constructed to determine the enzyme activities. One unit of glucanase activity was defined as the amount of enzyme that released 1 μmol of glucose per min under standard assay conditions.

2.6 Molecular weight determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% (w/v) polyacrylamide gel) was employed, to estimate the molecular weight of the purified enzyme, as described by Laemmli [19]. After electrophoresis, the gel stained with Coomassie Brilliant Blue R-250 and then destained with a solution containing methanol, glacial acetic acid, and distilled water (1:1:8). Molecular weight was estimated by comparing the relative mobility against those of reference proteins (MBI, Fermentas). Glucanase activity was analyzed as described by Regmi et al. [20].

2.7 Effects of pH and temperature

To determine the optimal pH for enzyme activity, enzyme assay was carried out at 50 °C using various buffers at pH ranging from 2.0 to 13.5. The buffers used were citric acid-sodium phosphate (pH 2–7.0), Tris-HCl (pH 7.0–9.5), sodium bicarbonate-NaOH (pH 9.5–11), and KCl-NaOH (pH 11–13.5). The optimal temperature for enzyme activity was determined at pH 7.5 by varying the temperature from 20 to 90 °C. To determine pH stability, the enzyme (100 mM)

enzyme was incubated in different buffers of varying pH at 4 ℃ for 24 h, and then the residual enzyme activities were measured under standard assay conditions. To determine thermal stability, the enzyme samples were pre-incubated at various temperatures up to 90 ℃ for 60 min, and the residual enzyme activities were evaluated under standard assay conditions.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Screening of bacterial strain for glucanase production

A process for selecting and screening microorganisms for the production of glucanase enzyme which is comprised by the steps of:

(a) forming a screening plate comprising a suspension of said microorganism on a solid medium which promotes the growth of said microorganism and the synthesis of glucanase enzymes thereby;

(b) incubating said inoculated medium under anaerobic conditions for a sufficient period of time to permit the growth of a screenable sub-population of microorganisms; and

(c) identifying in situ those colonies anaerobically expressing glucanase enzymes by detecting a zone of hydrolyzed starch surrounding each colony.

The presence of clear lytic zones around the colonies on the CMC agar plates indicates that CMC was utilized and degraded by a bacterial extracellular enzyme, which is present in CMC agar plate. Out of a total 78 isolates, only 22 formed clear lytic zones on mannan agar plates after incubation at 37 °C for 30 h, where the control had no effect (no lytic zones). The 22 strains were further cultured in galactomannan medium at 37 °C with shaking at 120 rpm for 60 h. As mention above, among the 22 strains, strain CSB34 showed the highest CMC-degrading activity, and thus was selected for further study.

A variety of approaches has been used to improve the economy of biologically-based industrial processes by "improving" the organism involved. These techniques constitute what may be categorized as strain improvement programs. The efficacy of improving said processes is dependent on the type of organism and the nature of the end-product.

The success of any strain improvement program will be directly affected by the facility with which genetic diversity can be generated in the subject organism, or alternatively the ease with which the genetic diversity already present in nature can be evaluated.

A colony that appears on agar medium following the plating out of spores, cells, or small hyphal fragments consists of a population most of which are genetically identical, although some cells may differ due to spontaneous mutation during the growth of the colony or to nuclear heterogeneity in the original propagule.

In a selection system, the experimental conditions are chosen so as to establish a growth differential between the rare strains possessing the desired characteristic and all other strains which do not possess said trait. In certain instances the selected strain will not grow under the conditions of the experiment while the non-selected strains will grow. Thus, by removing the growing strains by filtration or other means, the size of the population of cells remaining to be examined is dramatically reduced. Alternatively, conditions may be established such that the selected strain will grow while the non-selected strains are inhibited, here again effectively reducing the population to be examined.

3.2. Bacterial strain identification

Characteristics of the strain CSB34 were compared with those of known species. Morphological properties and biochemical characteristics suggested that the strain belong to the genus *Bacillus*. Furthermore, 16S rRNA gene sequence analysis verified that the parent strain was *Bacillus* sp. The highest identity was with *Bacillus subtilis* sub sp. *inaquosorum* (99.93%).

3.3 Identification of *Bacillus* strain

For molecular phylogeny, the 16S rRNA sequence of the local isolate was compared to a sequence of 10 *Bacillus* species. In order to determine the relation of the local isolate to these *Bacillus* strains, multiple sequence alignments weredone between the sequences of the 16S rRNA gene of various *Bacillus* species and local isolate. Computer assisted RNA searches against bacterial database similarly revealed that the 16S rRNA sequence was identical over 99% with those 10 *Bacillus* strains (Table 1).

16S rRNA sequence of *Bacillus* sp. CSB34

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGG

GAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAA

CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT

GGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCAC

TTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCAC

CAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA

CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG

CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT

CGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGG

GCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA

GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG

TAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC

AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGA

GTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACC

AGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC

GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT

GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCA

TTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA

TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA

CGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAG

GACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC

TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT

CTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA

ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG

GGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAG

GTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAAC

TCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG

GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGT

TTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGCCAGCCGCCGAA

GGTGGGACAGATGATTGGGGTG

Rank	Name	Strain	Accession	Pairwise Similarity (%)	Mismatch/Total nt
1	*Bacillus subtilis* subsp. *inaquosorum*	KCTC 13429	AMXN01000021	99.93	1/1472
2	*Bacillus tequilensis*	KCTC 13622	AYTO01000043	99.93	1/1472
3	*Bacillus subtilis* subsp. *subtilis*	NCIB 3610	ABQL01000001	99.86	2/1472
4	*Bacillus subtilis* subsp. *spizizenii*	NRRL B-23049	CP002905	99.8	3/1472
5	*Bacillus velezensis*	CR-502	AY603658	99.71	4/1403
6	*Bacillus axarquiensis*	CR-119	LPVD01000002	99.66	5/1472
7	*Bacillus mojavensis*	RO-H-1	JH600280	99.59	6/1472
8	*Bacillus vallismortis*	DV1-F-3	JH600273	99.59	6/1472
9	*Bacillus nakamurai*	NRRL B-41091	LSAZ01000028	99.59	6/1472
10	*Bacillus siamensis*	KCTC 13613	AJVF01000043	99.46	8/1472

Table 3.1. Similarity of 16S rRNA gene sequence of CSB34.

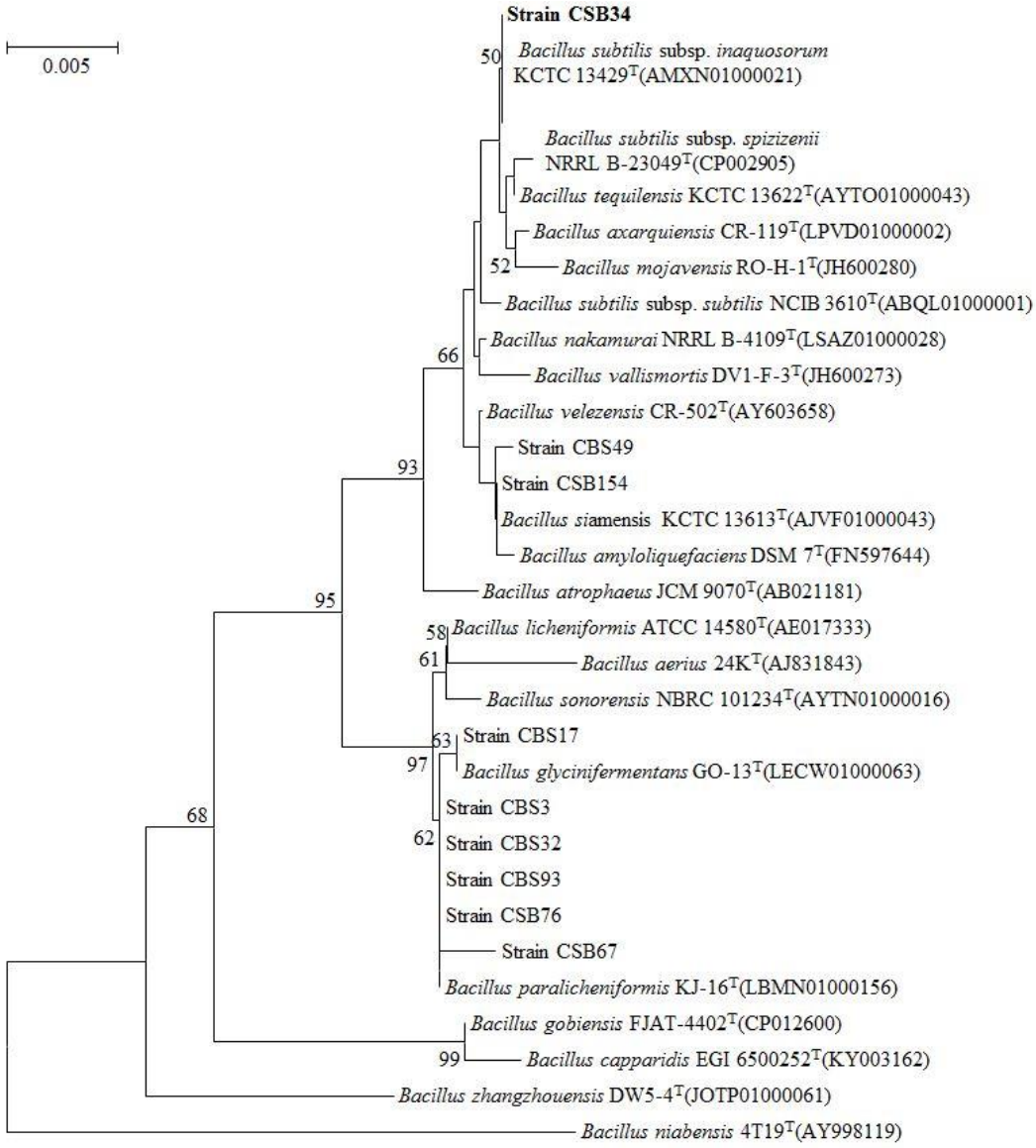
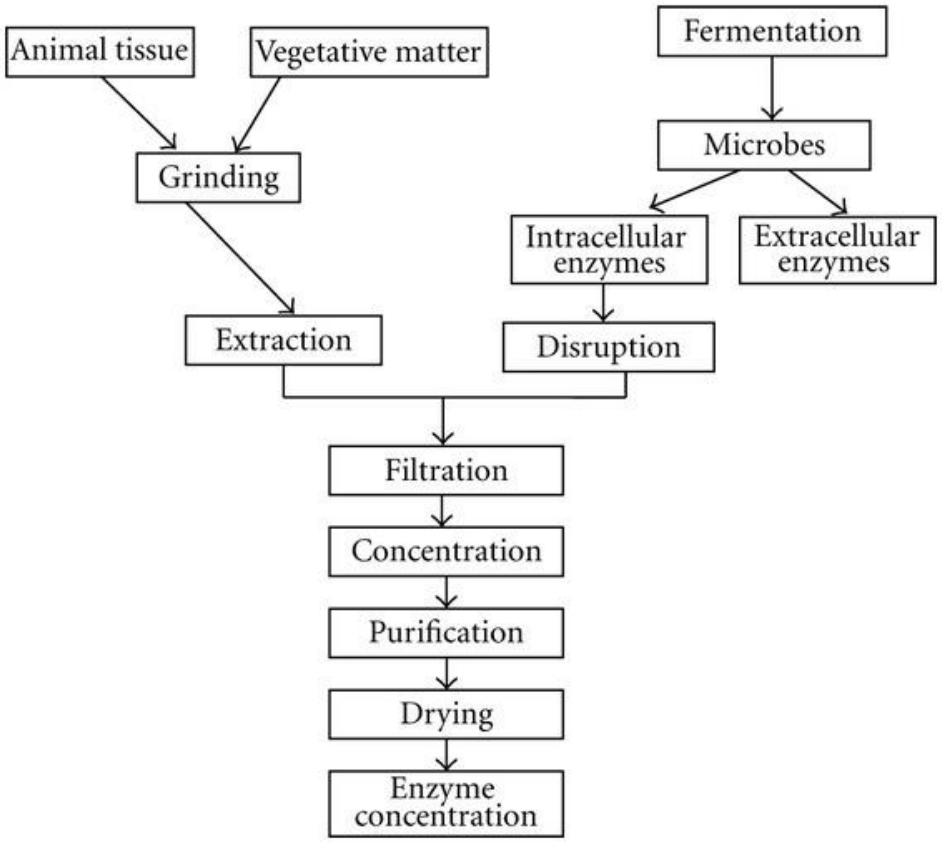


Figure 3.1: Phylogenetic tree of CSB34

3.4 Production of β-glucanase:

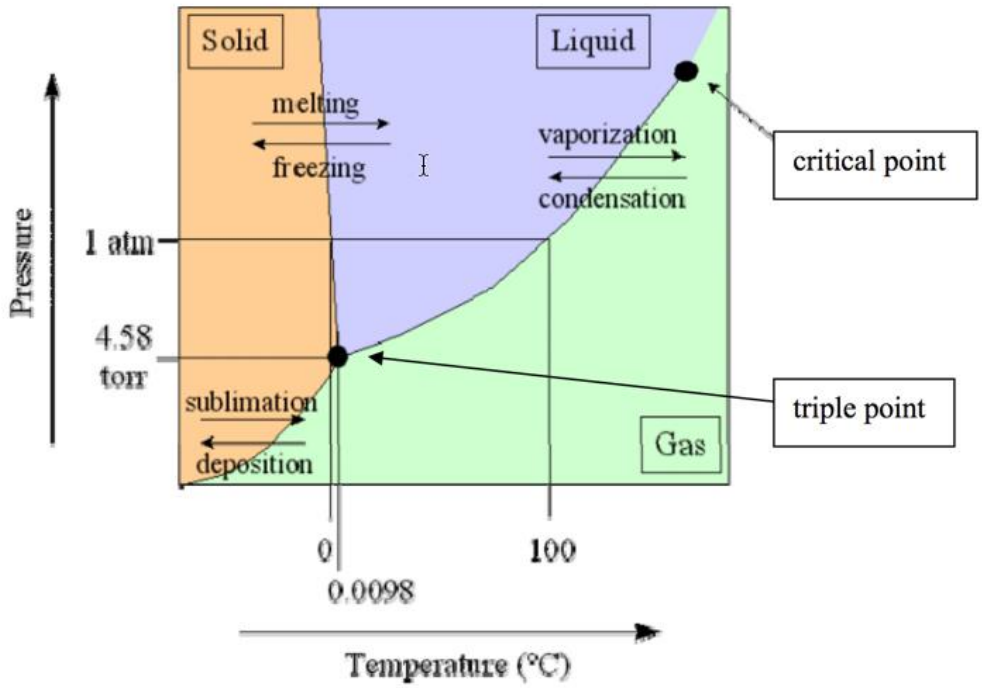
CSB34 was cultured in CMC medium at 37 °C with shaking at 120 rpm for 60 h. The glucanase was purified from the culture supernatant using ammonium sulfate precipitation, dialysis, and concentration followed by two steps of column chromatography, and gel filtration.

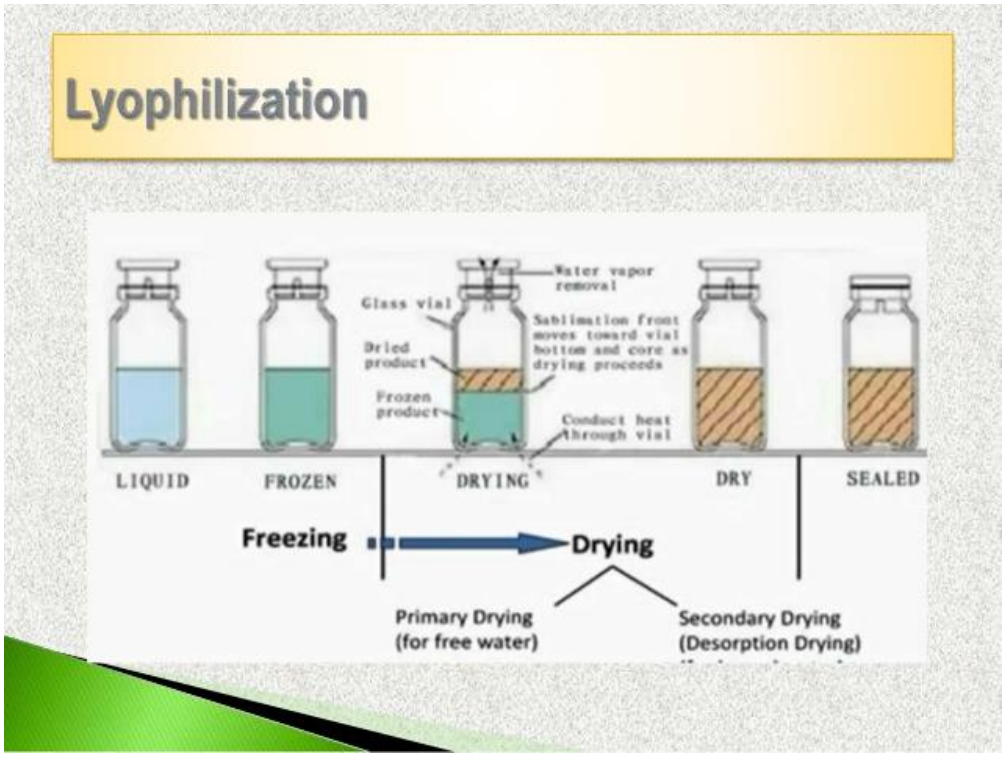


The beta-glucanase enzyme is manufactured by submerged fermentation of a pure culture of the *Bacillus* strain of CSB34. All settings were carefully designed, prepared, performed, cleaned and adjusted so as to escape contamination by any foreign microorganisms. During every steps

of fermentation, both physical and chemical control safeties were taken and microbiological experiments were conducted timely to ensure the sterility and ascertain production strain identity.

A new lyophilized stock culture vial of the *Bacillus* CSB34 production organism is used to start the production of every batch. Every fresh batch of the stock culture is strictly maintained for identity, sterility of the working areas, and enzyme producing ability before conducting each experiment.





Certain criteria fermentation batches including growth characteristics during fermentation are observed microscopically. Samples are examined from each fermentation stage such as inoculum, seed, and main fermenter, before inoculation time, at regular intervals of growth and before harvest of the cell culture. If microbiological contamination is determined, it will be rejected if deemed necessary. If the contamination is not so serious and is seen to be from common non-pathogenic environmental microbes, the fermentation may be processed and hence forth strict sterility is maintained.

3.5 Purification of Glucanase:

1. Ammonium sulfate precipitation method:

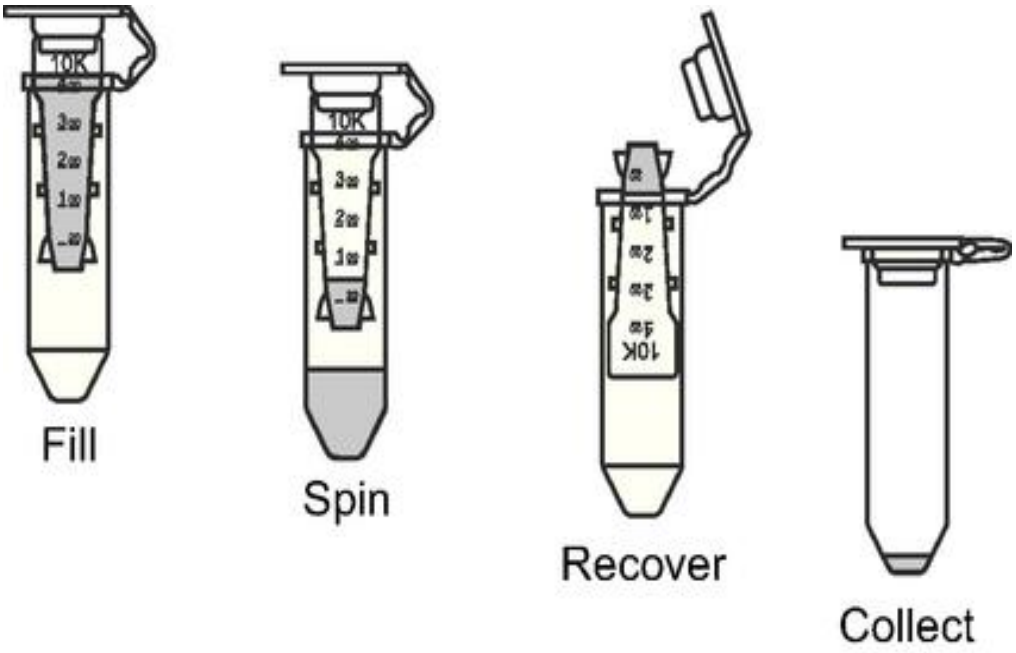
After 60 hours of growth, broth of the strain CSB34 was harvested. Cell free culture supernatant was precipitated using ammonium sulfate (30-80 % w/v) and stored at 4°C overnight with continuous stirring. The pellet was collected, suspended and dialyzed in Tris-HCl buffer (pH 7.5).

Initial concentration of ammonium sulfate (percentage saturation at 0 °C)	Percentage saturation at 0 °C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (g) to be added to 1 l of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

[a] Reprinted from Englard and Seifter (1990), which was adapted from Dawson et al. (1969).

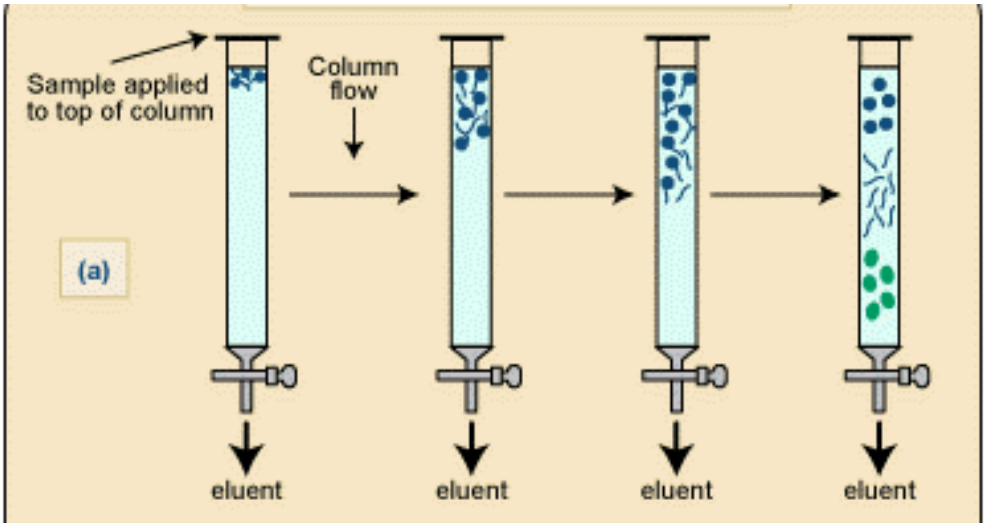
2. Ultrafiltration technique:

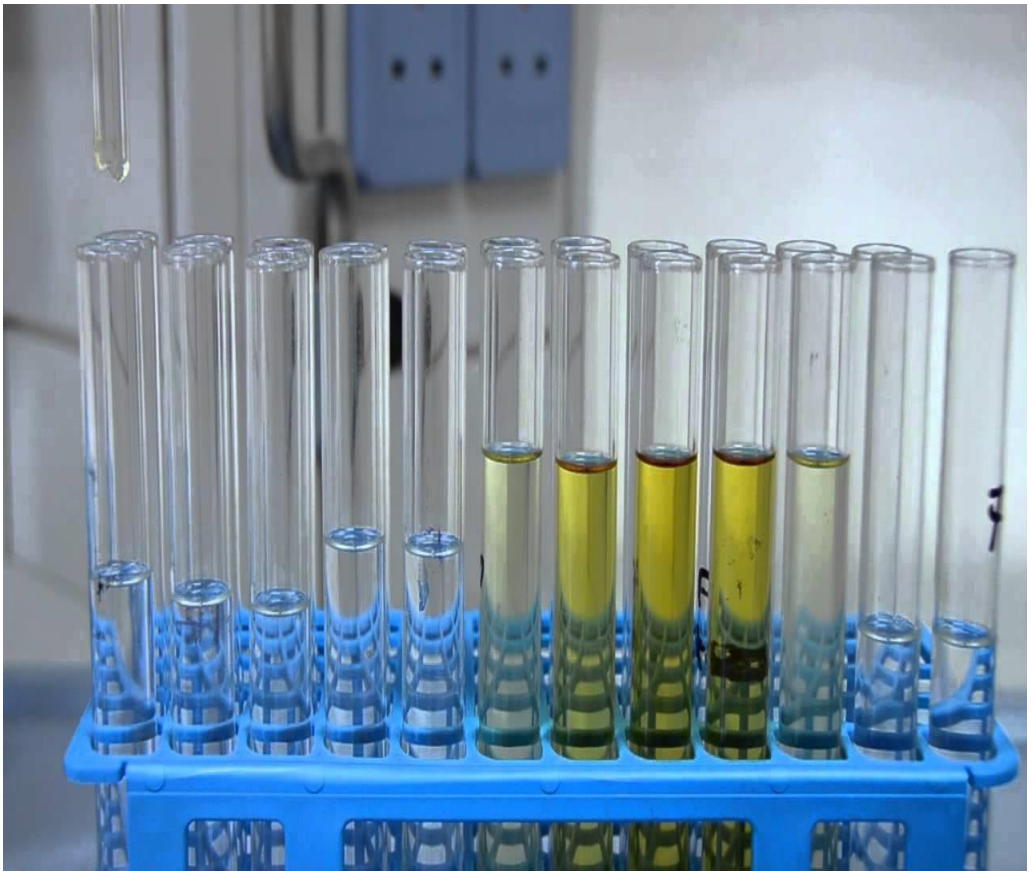
Centricone ultrafiltration technique was used after the ammonium sulfate precipitation method to purify the suspened pellets in buffer. 50 kDa centricone was used in this process respectively.

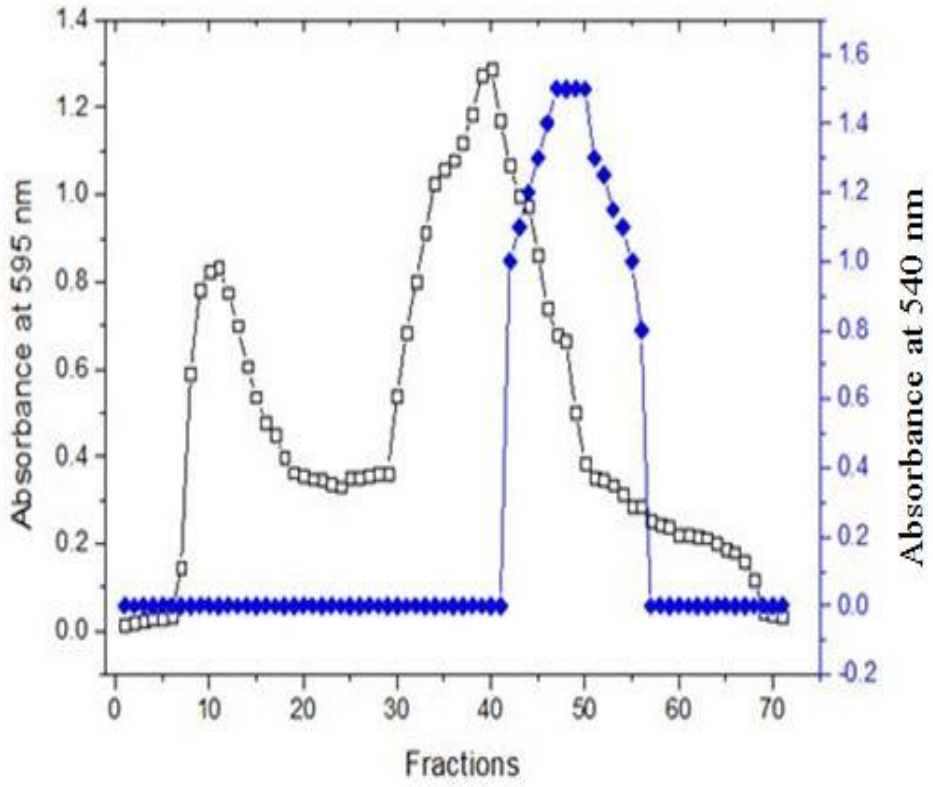


3. Column Chromatography:

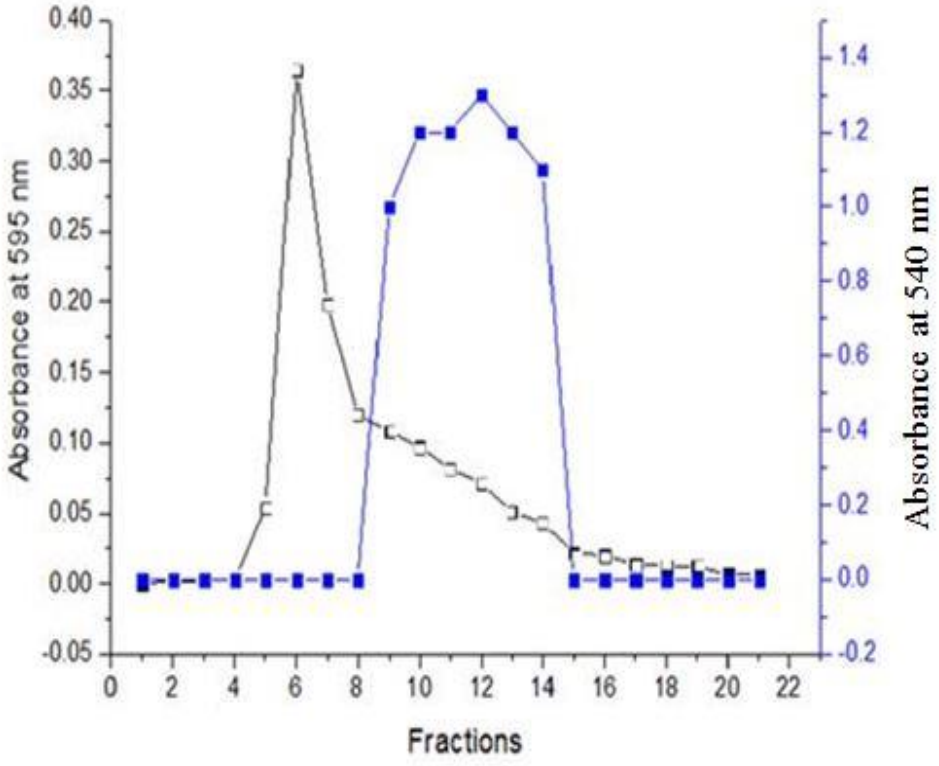
The crude extracts were applied to sepharose-cl-6b column, eluted with 10mM Tris-HCl buffer (pH 7.5) as a mobile phase. Fractions were collected and monitored for enzymatic activity. Active fractions were concentrated by lyophilizing, loaded onto a Sephadex G-50 column and eluted with the same buffer that was used before. The fractions positive for enzymatic activity were pooled and stored at 4ºC. Protein content was estimated using Bradford Method [17]. Figure gives the comprehensible concept of both chromatographic steps involved during purification and the selection of the active fractions by observing the enzymatic activity versus protein content plot.







A) Elution profile from Sepharose cl-6b

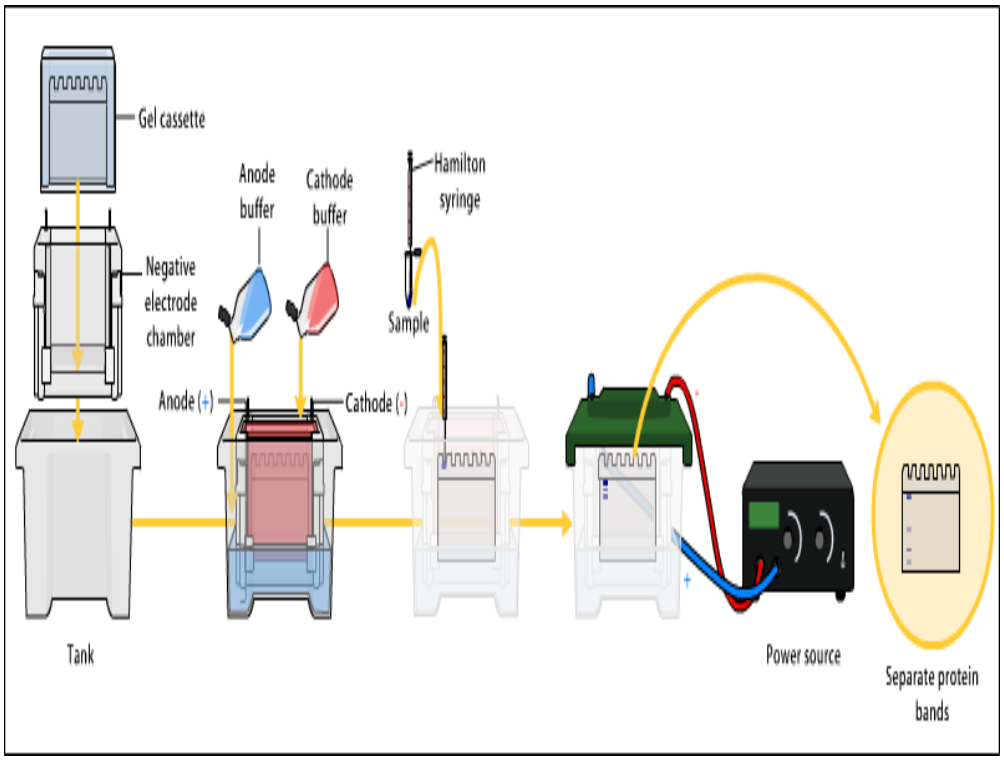


B) Elution profile of sephadex G-50

Figure 3.2: Elution plot from gel chromatography.

3.6 Determination of molecular weight

Purified glucanase was separated by 12.5 % SDS-PAGE. Molecular weight marker proteins were used to determine the approximate molecular size of active protein band. After electrophoresis, gel was stained with coomassie brilliant blue and destained with a destaining solution. The purified protein was confirmed by the presence of single band in gel having a molecular weight around 50 kDa.



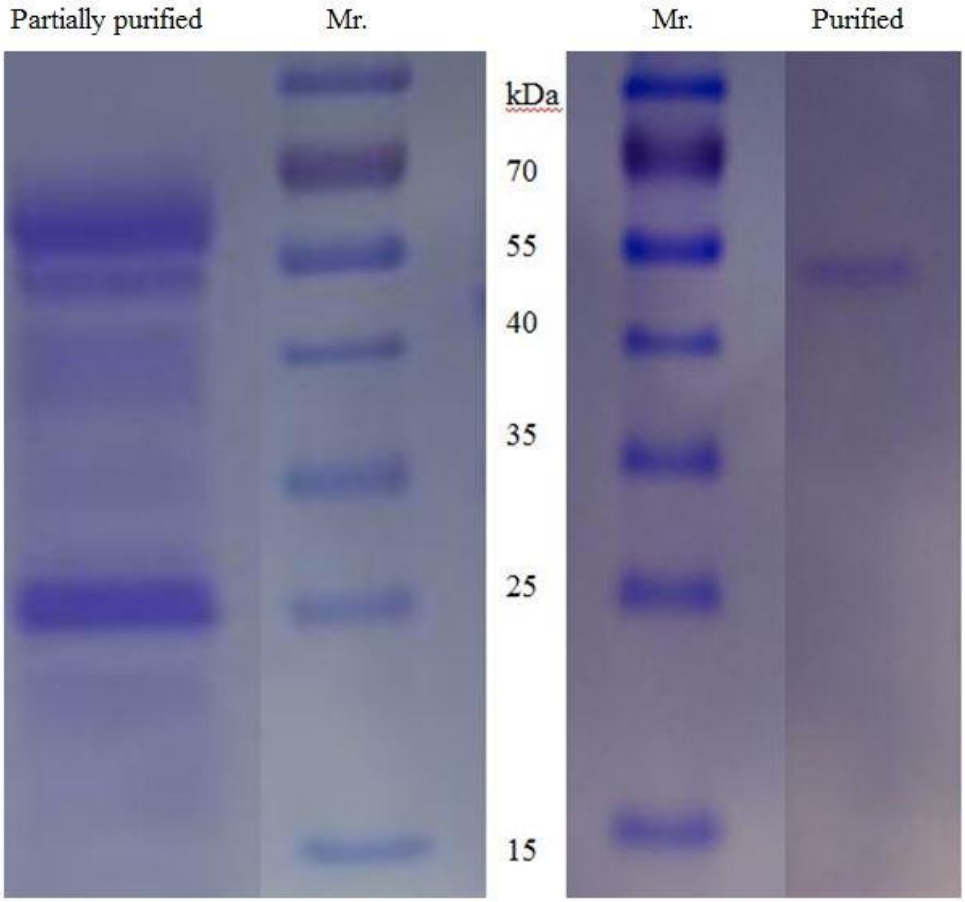
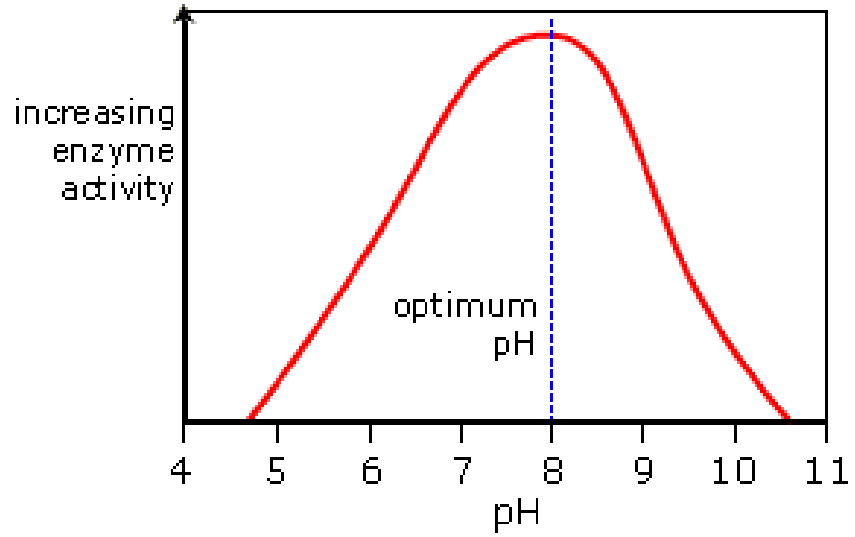
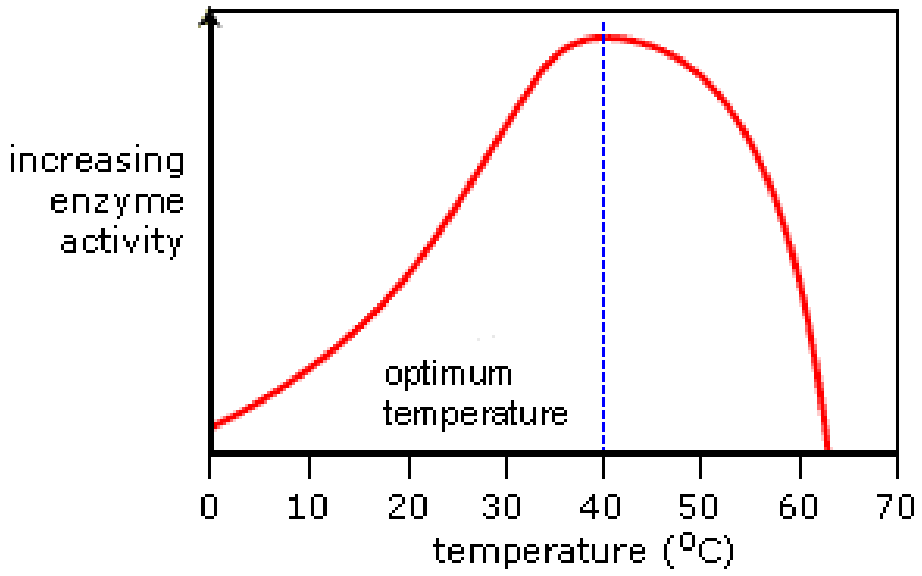


Figure 3.3. SDS-PAGE profile

3.7 Effects of temperature and pH





The influence of temperature and pH in the glucanase from strain CSB34 is represented in figure 11 and 12. It was found to be much more stable at temperatures as high as 60 ºC and loose about 25% of its activity at 70 ºC. The autoclaved sample lost its activity completely.

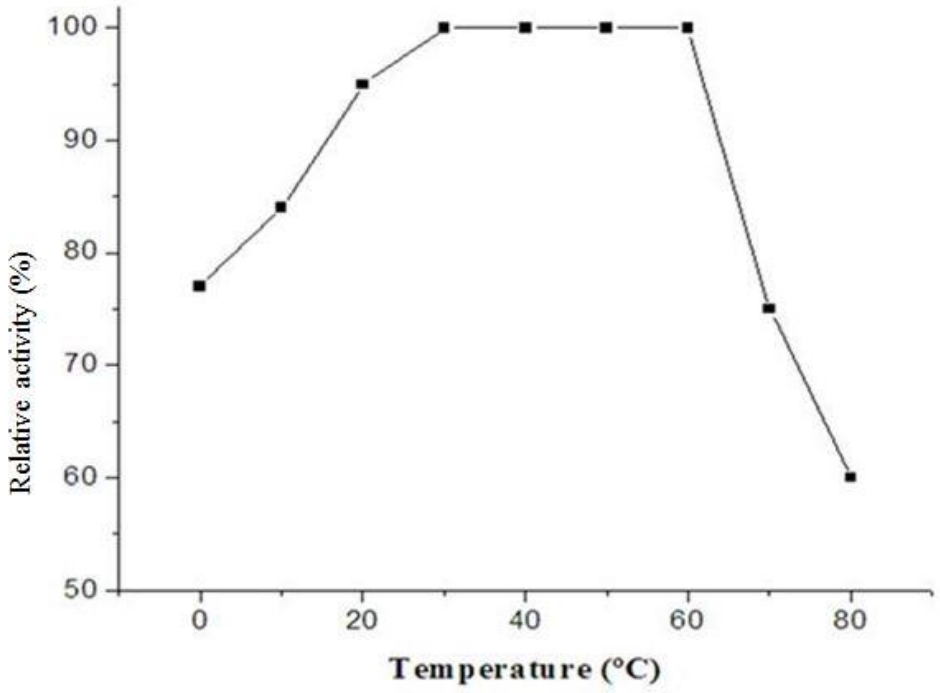


Figure 3.4. Effect of temperature

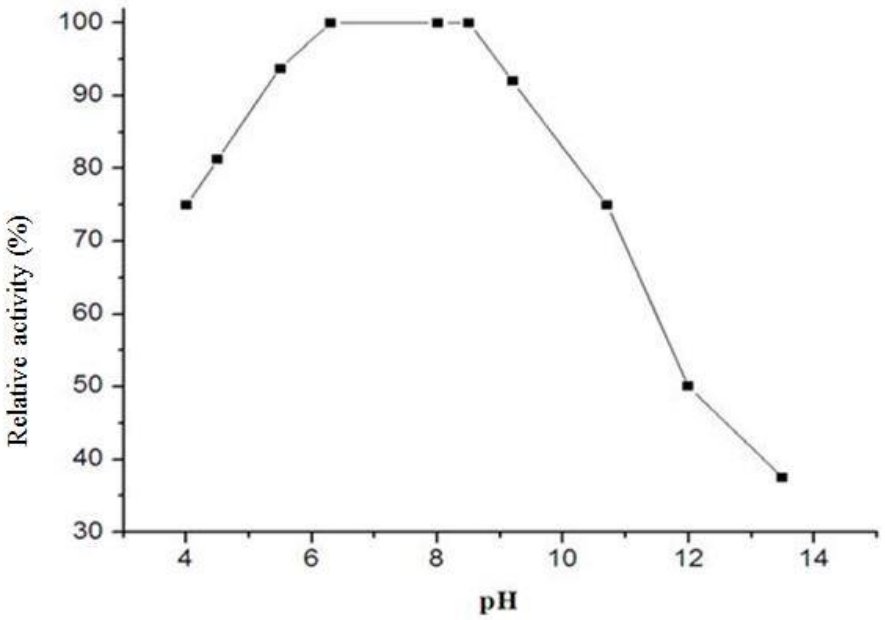


Figure 3.5. Effect of pH

Analysing the residual activity, it was seen that glucanase 34 was stable around pH 6.5-9. It lost 50% of activity when the pH was increased up to 12. Finally it showed 35% of residual activity at pH 13.5.

CHAPTER 4: CONCLUSIONS

In this study, a potent microbial enzyme (glucanase) was produced from a microbial strain and identified as *Bacillus* sp from 16S rRNA gene sequences analysis. This study aimed to determine the enzymatic activity of Glucanase34 with characterization. Optimized media, CMC media was found as the suitable nutrient sources for the maximum enzyme production. Glucanase34 was purified using Sepharose Cl-6B and Spehadex G-50 gel filtration column chromatography respectively. It was found to be stable over wide range of temperatures up to 60ºC and pH (6.5-9) conditions.

The molecular weight of Glucanase34 was found to be 50 kDa. The wide range of pH stability and high temperature signifies the microbial enzyme has the capacity to be developed as an industrial enzyme.

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