



IDENTIFICATION AND CHARACTERIZATION OF PROTEASES AND AMYLASES PRODUCING *Bacillus licheniformis* STRAIN EMBS026 BY 16S rRNA GENE SEQUENCING

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Abstract- Development of natural antimicrobial strategies has always attracted researchers, as disease outbreaks are recognized as important constraints to living organisms because of the development of antibiotic resistance in microorganisms. Use of probiotic bacteria has been in practice as alternatives to antimicrobials in disease control as microbial control agents. Probiotics, the natural and beneficial micro flora has been widely accepted and used in farming and aquaculture because of its diversified applications. It improves water and pond sediment quality which in turn improves aquatic life. Bacterial pathogenicity and virulence can be controlled without using excessive antibiotics which help to minimize the risk of multiple antibiotic resistances. Probiotic help in increased productivity and profits when used properly as they produce antagonistic compounds that are inhibitory toward pathogens, compete with harmful microorganisms for nutrients and energy, compete with deleterious species for adhesion sites, enhance the immune response of the animal, improve water quality, and interact with phytoplankton. Among the large number of probiotic products in use today are bacterial spore formers, mostly of the genus *Bacillus*. The genus *Bacillus* is widely used as probiotic products because of its extremely resistant spores which provide exceptional longevity and release exoenzymes. The current investigation is to identify a novel strain of *Bacillus licheniformis* by application of 16S rRNA gene sequencing. The approach is to identify a novel, proteases and amylases producing bacteria which is used for detergent production. The sample was isolated from near Gudiwada, Krishna district, Andhra Pradesh, India. Subsequently the sample was serially diluted and the aliquots were incubated for a suitable time period following which the suspected colony was subjected to 16S rDNA sequencing. The results showed the isolate to be a novel, high alkaline protease producing bacteria, which was named *Bacillus licheniformis* strain EMBS026, after characterization the sequence of isolate was deposited in GenBank with accession number JQ267798.

Key words- 16s Gene sequencing, *Bacillus licheniformis* strain EMBS026, Proteases and amylases producing bacteria.

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Introduction

Probiotics are live microorganisms introduced into the body for its beneficial qualities. They increase host fitness by balancing the intestinal micro flora, inhibiting the growth of pathogenic bacteria, promoting good digestion, and boosting immune function. According to the currently adopted definition by FAO/WHO, probiotic are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" [1]. Probiotics are typically broken down into two types. The first of these types are those that contain lactic acid bacteria and the second type of com-

monly used probiotic are Non-lactic acid bacteria. *Bacillus licheniformis* is a gram-positive saprophytic bacterium ubiquitous in the environment and play vital role in cycling nutrients. It has been used for several decades for the industrial production of alpha-amylase and classified as GRAS (Generally Recognized As Safe) by the U.S. Food and Drug Administration. According to Eveleigh (1981), the main industrial protease was one produced by *B. licheniformis* for use as a cleaning aid in detergents. Laccases are industrially relevant enzymes and catalyze wide range of reactions due to their broad substrate spectrum

which includes cross-linking of phenolic compounds, degradation of polymers, ring cleavage and oxyfunctionalization of aromatic compounds. Unlike many other oxidoreductases they do not require cofactors like NADH or NADPH. Laccases are commonly used for pulp-pretreatment in the paper production process, dye bleaching in the textile industry, detoxification of xenobiotics, organic synthesis, and bioremediation [2]. Laccases are very common among bacteria [3], fungi, insects [4-5] and higher plants [6]. Fungal laccases are applied in various processes like lignin degradation [7], pigment production [8], and plant pathogenesis [9]. Plant laccases have a major role in the lignification process [10]. The physiological function of bacterial laccases is still unclear, but it is believed that they play a role in melanin production, spore coat resistance, morphogenesis and detoxification of copper [11].

Biological activity of *Bacillus licheniformis*

Bacteria of the *Bacillus* genus are omnipotent in nature, predominant in soil, and these bacteria have also been isolated frequently from water and air. Large amounts of bacilli (10^7 - 10^8 cfu) consistently enter the gastrointestinal and respiratory tracts of healthy people through air, water, and food and exist in the gut microflora. Some researches showed that *Bacillus* organisms are a normal component of human intestinal microflora. Use of *Bacillus licheniformis* increase productivity in aquaculture along with lowering down of a large variety of pathogens and bacterial resistance which eventually prevent bacterial diseases in aquaculture. The micro flora *Bacillus pumilus* and *Bacillus licheniformis* isolated from the rhizosphere of alder (*Alnus glutinosa* [L.] Gaertn.), reported to have a strong growth-promoting activity. It has been documented that the culture of both bacteria accumulates bioactive C19-gibberellins in relative high amounts and that these physiologically active bacterial Gas could mediate the promotion of stem elongation induced by the PGPR in the host plant.

Three strains of *Bacillus licheniformis* (A12, D-13, and M-4) reported to have marked amoebicidal and amoebolytic activity against human pathogenic and nonpathogenic strains of *Naegleria* [12]. Strain A12 produced two amoebolytic peptide antibiotics [13]. Moreover, three amoebolytic peptides (named amoebicins m4-A, m4-B, and m4-C) different from those produced by strain A12 were isolated from the spent medium of *B. licheniformis* M-4 and characterized [14]. A new in-feed probiotic, LSP 122 (Alpharma), containing 10^7 viable spores of *B. licheniformis* per gram of feed was tested for its efficacy to control Post-weaning diarrhoea syndrome (PWDS) of piglets, caused mainly by enterotoxigenic *Escherichia coli* (ETEC) strains. The results showed that in-feed supplemented with probiotics is a very useful agent for the control of PWDS due to ETEC. The mode of action of probiotic LSP 122 against post-weaning diarrhoea syndrome of piglets is not fully known [15]. *Bacillus licheniformis* significantly decreased *Vibrio* counts and increased phenoloxidase and superoxide dismutase activities when administered to the white shrimp (*Litopenaeus vannamei*) by improving its immune ability [16]. It has been documented that supplementing ewe's feed with probiotics containing *Bacillus licheniformis* and *Bacillus subtilis* have beneficial effect on young lamb mortality, sheep milk production, fat and protein content when administered in the late pregnancy and lactation feed of ewes [16].

Importance of Protease and Amylase

Proteases are the most important industrial enzymes accounting for about 50% of the total industrial enzyme market [17]. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. Proteases and amylases are used together in many industries such as food industry, pharmaceuticals, detergent industries, etc. Detergent formulations are fortified with both proteases [18] and [19] and amylases [20] individually. In view of the recent trend of developing environment friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes.

The industrial demand of highly active preparations of proteolytic enzymes with appropriate specificity and stability to pH, temperature, metal ions, surfactants and organic solvents continues to stimulate the search for new enzyme sources. Proteases with high activity and stability in high alkaline range and high temperatures are required for bioengineering and biotechnological applications. Their major application is in detergent industry, because the pH of laundry detergents is generally in the range of 9.0-12.0, accounting for about 35% of the total microbial enzymes sales [23]. Bacteria, moulds and yeasts are some of the microorganisms that produce proteases. Most of the commercial alkaline proteases were isolated from *Bacillus* species [24]. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

Many *Bacillus* serine-proteases have been characterized and their encoding genes have been cloned and sequenced [25], [26] and [27]. Subtilisin Calsberg produced by *B. licheniformis* [25] and Subtilisin Novo produced by *B. amyloliquefaciens* [26] have been the enzymes of choice in detergent formulations. Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermo tolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to the natural plant proteinase inhibitors and is therefore useful in the brewing industry. The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Hydrolyzates which inhibit the angiotensin I-converting enzyme (ACE) are rich in acidic amino acids, poor in hydrophobic ones, and effective for use as a physiologically functional food material by virtue of little bitterness, a fish odor and powerful ACE inhibitory activity were prepared from sardine muscle by *Bacillus licheniformis* alkaline protease. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteinases, which are not affected by chelating agents.

It can be used to produce thermo stable alpha amylase which is

active at relatively low pH and low calcium concentration which can be used further for starch hydrolysis conducted at high temperature (Starch liquefaction in the production of nutritive sweeteners from starch). Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure-function relationship, in the synthesis of peptides, and in the sequencing of proteins.

Source of Isolation

The microorganism used was an alkaliphilic bacterium, which was isolated from *pond water* near Gudiwada, Krishna Dist. Andhrapradesh, India. It was identified as *Bacillus licheniformis* strain EMBS026.

Use of Probiotics in Pond Water

Research conducted in last decade has proved that probiotic can enhance water and soil quality, improves production, and increase profits. (28) A number of biological products have been used as water and soil quality conditioners in aquaculture ponds and particularly in shrimp ponds. Several studies have demonstrated higher survival of fish and thus greater net production in ponds when treated with live *Bacillus* inoculate but the mechanism by which the bacteria improved survival is unknown. Pond studies also showed that applications of an enzyme preparation tended to enhance microbial mineralization of organic matter, but no effect on net fish production was observed. An extract of grapefruit seed caused greater survival and higher production of shrimp. Again, the mode of action is unknown because water quality was not measurably improved. These few studies suggest that probiotics possibly can be beneficial in aquaculture ponds. Too little is known about their modes of action, the conditions under which they may be effective, and application rates and methods for general recommendation of their use. Nevertheless, the products are safe to humans and the environment, and their use poses no hazards.

Sample Collection and Storage

A water sample was collected from a *pond near* Gudiwada, Krishna Dist. Andhrapradesh, India. The site was surveyed before water sample collection and then the sample carrying the suspected probiote was collected from five different sites from the chosen pond. It was important to collect the sample from deep inside the pond and not from the surface; the microbial community would be more diverse one foot below the surface than on the surface. Thus the chances of collecting the desired probiote along with the sample would be more if the sample is collected from one foot below the water surface. Sample should be collected from a location which is deep enough and away from sources of water. Two type bottles were used to collect samples for analysis. The largest sample bottle with the yellow label is used for all chemical analyses and the clear bottle with the green label has been sterilized to prevent bacterial contamination used for bacteria analysis. We did not touch or lay the lid down on the ground to prevent bacterial contamination on the inside of the bottle or cap. After rinsing, submerge the bottle below the water level and allow it to fill completely to the top marked "100 mL". Screw the lid on tightly to prevent leakage. Place the bottle in the bubble wrap sleeve and refrigerat-

ed (4°C) till further analysis. The samples instead of being frozen are simply refrigerated since the refrigeration temperature of 4°C is sufficient to temporarily suspend the metabolism of the microbes present therein. Water samples analyzed within 30 hours of collection for bacterial analysis to produce accurate results.

Sample Mixing and Serial Dilution

10 ml of water was taken from each of the five samples and samples were mixed thoroughly. This suspension was serially diluted following the usual method for serial dilution. We took six - 15 ml falcon tubes with 9 ml of sterile buffer and label each tube to reflect a ten-fold dilution of the previous tube (i.e. tube 1 = 10^{-1} , tube 2 = 10^{-2} , etc.). Using a 1 ml pipette, transfer 1 ml of pond water sample to the first dilution tube (10^{-1}), briefly vortex the tube and transfer 1 ml of the suspension to the second dilution tube (10^{-2}). We continued this dilution until the original sample is diluted to 10^{-6} .

Isolation by Spread Plate Method

Samples from three consecutive dilutions (10^{-6} to 10^{-4}) were plated in triplicate to ensure that countable plates (between 30 - 300 colonies) will be generated. We started with the lowest dilution. Therefore, the same pipette tip was used for each of the nine inoculated plates (three dilutions x three replications). If the tip has contacted any surface, replaced it with a new one. Transfer 100 μ l of the highest dilution to be plated to the surface of each of three Petri dishes containing sterile agar media. Sterilized glass rod soaked in 70 % ethanol and treated with heat and to be used as spreader. The alcohol will kill any bacteria on the glass surface and the flame will burn-off the alcohol. Touch the spreader to the agar surface to cool the glass. Using a back-and-forth motion, spreaded the inoculant over the surface of the agar and Incubated the plates at 25 °C for 24-48 hours. After the incubation is complete, identify the dilution that resulted in the growth of bacterial colonies between 30 - 300. Count the colonies and write the colony number on the plate.

Culturing of the Bacterial Isolate

Isolated CFU's were routinely grown in Luria-Bertani (LB) broth medium (composed of (g/l): peptone, 10; yeast extract, 5; NaCl, 5 [30]). Media were autoclaved at 120 °C for 20 min. Cultivations were conducted in 25 ml of medium in 250 ml conical flasks maintained at 37 °C. Incubation was carried out with agitation at 200 rpm for 24 hr. The cultures were centrifuged and the supernatants were used for isolation of DNA for gene sequencing.

DNA Isolation from Cultured Bacterial Cells

B. licheniformis strain EMBS026 was isolated from *pond water* near Gudiwada, Krishna Dist. Andhrapradesh, India. Sample collected was plated onto skim-milk agar plates containing (g/l): peptone 5, yeast extract 3, bacteriological agar 12 and skim-milk 250 ml, pH 10.0. Plates were incubated 24-48 h at 37 °C. A clear zone of skim-milk hydrolysis gave an indication of protease-producing strains. Individual colonies were purified through repeated streaking on fresh agar plates. Among many strains, showing different proteolytic activities, isolate EMBS026 was selected. The strain was identified as *B. licheniformis* according to the methods described in Bergey's Manual of determinative Bacteriology

[29] and on the basis of the 16S rDNA sequence analysis. After completing the protocol, it is very necessary (especially in this case since it is not the standard protocol) to confirm if or not is the isolated material actually DNA. For this purpose we make use of Gel Electrophoresis. Thus 15µL of substance was mixed with 2µL of gel loading dye and this mixture was loaded into a well cast beforehand in the gel (actually since to minimize the chances of failure of the experiment, we carried out the entire experiment taking the culture in four identical vials, thus the sample-gel loading dye mixture was also loaded into four wells). The gel was lifted and viewed under UV transilluminator after allowing for 15 min of run approximately [Fig. 1].

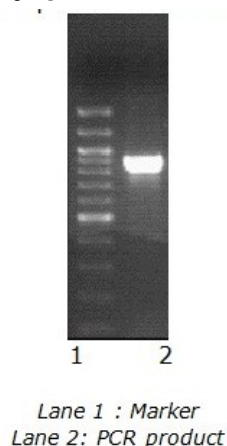


Fig. 1- 16S rRNA PCR Product on 2% Agarose Gel.

PCR Amplification of the 16S rRNA Gene

The gene coding for the 16S ribosomal RNA from the isolated DNA was amplified across 25 cycles, using the "Corbett Research Ltd, Gradient Thermal Cycler" machine. *Pre-Denaturation*, in which the entire amount of isolated DNA is separated into single strands. This process was carried out at 94°C for 5 minutes. *Denaturation* was carried out at 94°C for 1 minute in each PCR cycle. Since in the denaturation steps of each cycle after the denaturation, only the double stranded target gene of our interest was separated into single strands, this step was carried out only for one minute in each cycle. *Annealing*, here the single stranded primer gets attached on to its complementary single stranded sequence (16S rRNA gene, in this work). It was carried out at 52°C for 1 minute in each cycle. *Renaturation* was carried out at 72°C for 1 minute in each cycle. The *Final elongation* was carried out at 72°C for 7 minute after all 25 cycles were over. This step is important to extend any remaining piece of single stranded DNA. The one very big advantage in using the 16S rRNA gene for molecular characterization of bacteria and identification of new bacterial species/strains is that even though the sequence of the 16S rRNA coding gene of the isolate is yet to be sequenced, as a matter of fact is the very motive behind some research works like ours, it is possible to design primers for the PCR amplification of such yet to be sequenced genes too. Such primers are designated as "Universal Primers". The idea behind the usage of universal primers is that the 16S rRNA gene is unique for different species as well as for different strains of the same species but the flanking regions of the 16S rRNA gene remains highly conserved across different species. Therefore the primers can be designed for a

novel species also since it would have the same flanking regions of its 16S rRNA gene, and so the primer will attach to these flanking regions and facilitate the extension of the gene by the respective DNA polymerase enzyme. The following were the primers used for the amplification and the PCR product size were 1.4 kb.

27F : 5' - AGA GTT TGA TCC TGG CTC AG -3'
1391R : 5' - GAC GGG C(AG)G TG(AT) GT(AG) CA -3'

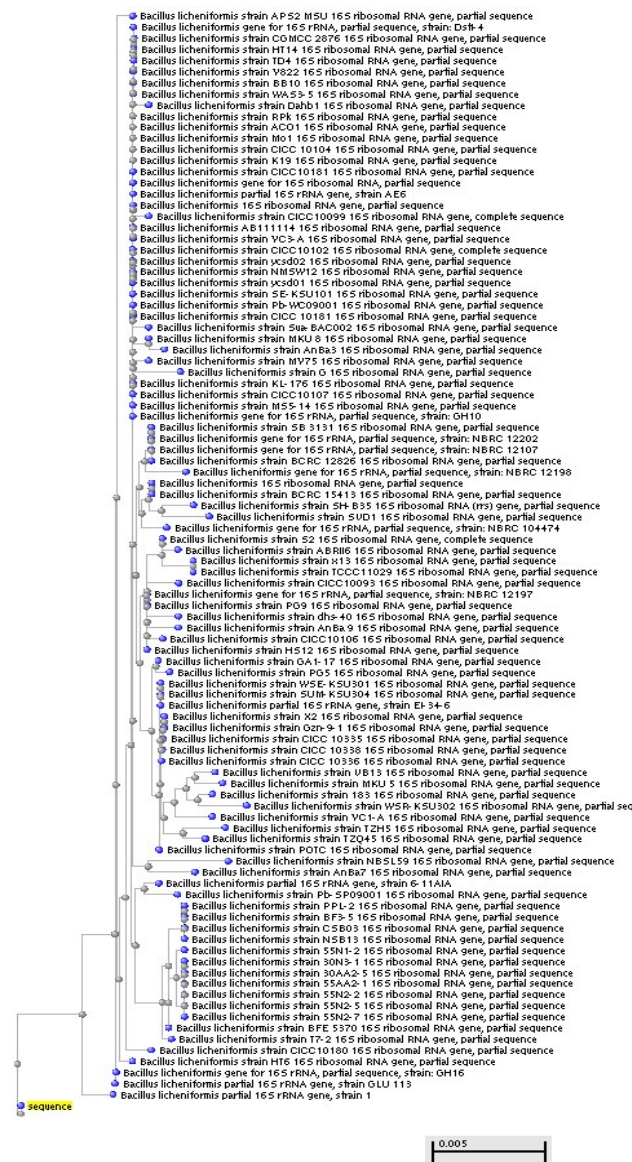


Fig. 2- Phylogenetic affiliation of *Bacillus licheniformis* strain EMBS026 against other species of *Bacillus licheniformis*

The PCR reaction mixture used had four components in it. It contained the PCR master mix(20 µL), which consisted of Taq polymerase enzyme, dNTP's and 10X PCR buffer in it; the template (2µL); the forward and reverse primers(1µL each); distilled water (6µL). All these components were added to a 200L capacity PCR vial in the sequence as described above and in the respective amounts.

After adding all the above listed components in the above listed

sequence to the 200 µL PCR vial, the vial was placed in a 1500 µL capacity bigger vial and subjected to a very brief spinning in centrifuge, only for the purpose of proper mixing of the contents.]. It was shown by Woese and others that phylogenetic relationships of bacteria could be inferred by comparing a stable part of the genetic code. Such stable regions in bacteria are the genes that code for the 5S, 16S and 23S ribosomal RNA and the spaces between these genes. The part of the DNA, now most commonly used for taxonomic purposes with regards to bacteria is the 16S rRNA gene [Fig. 2].

Sequencing of the PCR Product

The PCR product was sequenced using the ABI Prism. Sequencing reactions were carried out with ABI PRISM Dye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems Inc., USA). The novel isolated sequence was deposited in GenBank with accession number JQ267798, maintained by the National Centre for Biotechnology Information (NCBI), at the National Institute of Health (NIH), Rockville, Maryland, USA.

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