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Purification, Immobilization, of amylase from *Bacillus licheniformis* and its application in Biodegradation of food waste

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ABSTRACT
The aim of this study was to identify a soil bacterial strain that produces α-amylase, followed by its purification and immobilization for domestic kitchen food waste treatment. The domestic food waste treatment includes grains, vegetables and fruits that closely resembled the food waste discharged from domestic kitchens. Results showed that B. licheniformis possess an excellent amylolytic property. Highest α-amylase activity was obtained when the isolate was cultivated at pH 7 (1.45±0.54 U/ml), temperature at 37° C (1.19±0.06 U/ml) and starch at 4% (1.21±0.2 U/ml). Dialysis and ammonium sulphate precipitation were used for partial purification. At each stage, the amylase assay, protein concentration, and specific activity were analyzed. It was observed that increase in specific activity was inversely proportional to protein concentration values. The 60% saturation of ammonium sulfate gave a yield of 0.4±0.05% having specific activity of 3.96±0.13U/mg. The acquired ammonium sulfate precipitate was subjected to dialysis, and then the specific activity reached up to 6.10±0.34U/mg, with purification fold 1.48±0.07and yield of 0.32±0.02%. Since, immobilization plays an important application in industries, the physical immobilization of amylase in sodium alginate showed satisfactory activity and stability for B. licheniformis (1.48±0.11U/ml). The immobilized amylases exhibited the largest reduction of food waste at a temperature of 37°C, resulting in a weight loss of about 83%±0.03 of solid content. Throughout the study, B. licheniformis produced satisfactory results. Hence, the organism was sequenced and phylogenetic studies were carried out. The results showed that they share a common ancestor with five distinct Bacillus species. The findings showed that the B. licheniformis isolate possessed degradation of food wastes at faster rate with α-amylase.
Keywords: *Food microbiology, α- amylases, Purification, Immobilization, Biodegradation, Phylogenetic analysis*

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INTRODUCTION

Starch is broken down by the enzymes called amylases. They catalyze the hydrolysis of internal 1,4-D-glycosidic bonds in polysaccharides while maintaining the anomeric structure in the end products. The majority of amylases belong to the hydrolase group of enzymes, which are metallo-enzymes that rely on calcium ions (Ca^{2+}) for their function [1]. Amylases have been demonstrated to be present in microbes despite being prevalent in both plants and animals. Microorganisms have been shown to have two significant amylase types: gluco-amylase and α-amylase. In the linear amylose chain, 1, 4-a-D-glucosidic linkages between neighboring glucose units are randomly broken by amylases (endo-1, 4-a-D-glucan gluco hydrolase, E.C. 3.2.1.1) [2]. A step-by-step process is used by gluco-amylase (exo-1, 4-a-D-glucan glucano hydrolase; E.C. 3.2.1.3) to hydrolyze individual glucose units from the non-reducing ends of amylose and amylopectin [3]. Amylases play a significant role in 25–33% of the world enzyme market [4- 5]. The breadth of the amylases has expanded in numerous fields, including clinical, pharmaceutical, and analytical chemistry. In addition to starch saccharification, they are used in the baking, brewing, detergent, textile, paper, and distilling sectors [6]. The starch hydrolyzing enzymes and starch modifying, or trans-glycosylating, enzymes make up roughly two groups in the amylase family [7]. In the starch processing industry, enzyme hydrolysis is preferred over acid hydrolysis for a number of reasons, including reaction selectivity, product stability, low energy requirements, and lack of neutralization [8].

The most prevalent bacteria found in soil are gram-positive, aerobic, rod-shaped endospore-forming *Bacillus* bacteria [9]. They produce industrial enzymes like proteases, α-amylases, and other macromolecular hydrolases prolifically. This is based on the fact that in their natural environment, the

ability to decompose and use soil debris, particularly that generated by plants (protein, starch, pectin, cellulose, etc.), provides essential nutritional sources. Due to their rapid fermentation cycles, capacity to release proteins into extracellular medium and overall handling safety, the *Bacillus* species comprise microorganisms that are important for industry [10]. Each use of amylase requires certain characteristics in terms of specificity, stability, temperature, and pH dependency [11]. The amylase has demonstrated activity over a wide temperature range, which is useful as it can lead to its application as an additive in detergents and in textile design [12].

Covalent binding is a typical method for immobilization; it can be performed via direct association with the enzyme and the substance through the covalent bond. Covalent technique of immobilization is generally employed when a reaction process does not require enzyme in the result, this is the criteria to choose covalent method [13]. Enzymes are physically bound to the surface of an inert support during adsorption. Alginate is a natural polysaccharide that is synthesized by brown seaweeds and by soil bacteria [14]. It is the first by product of algae purification; it is the most commonly employed alginate type in the industry. In addition to segments of alternating guluronic and mannuronic acids, sodium alginate contains -L-guluronic acid residues (G blocks) and ß-D-mannuronic acid residues (M blocks) (GM blocks) [15-16].

The Food and Agricultural Organization FAO (2015) reported that about 1.3 billion tonnes of the world's yearly food production for human consumption are wasted at various phases, from the original production stage to the supply chain and ultimate home consumption [17]. By introducing mesophilic aerobic and anaerobic fermentation to treat food waste, Kiran et al., (2014) and Kibler et al., (2018) demonstrated that microbes can consume and reduce/biodegrade the total quantity of food waste released from both commercial and home sources [18-19]. Furthermore, according to An et al., (2018), Awasthi et al., (2017), and Awasthi et al., (2018), *Bacillus* spp. is a rapidly proliferating bacterium that possesses potent extracellular enzymes that have varying effects on hydrolyzing organic compounds and decomposing food wastes with the help of the degradative enzyme activity [20-22].

In the present research, an extracellular, α-amylase producing *Bacillus* was isolated and purified from rhizosphere soil. Ammonium salt precipitation and dialysis were performed has a purification step. Amylase assay and protein concentration were measured at each stage, which increases the specific activity of the enzymes and makes them more specialized for commercial uses. Purified amylase was immobilized using sodium alginate, and the features of the immobilized enzyme, such as activity and stability, were investigated. Immobilized enzymes were used for biodegradation of food waste by using standardize food waste formula.

MATERIAL AND METHODS

Isolation and identification of the organism

The termite soil sample was collected from Gandhi Krishi Vigyana Kendra (GKVK), Bengaluru. Serial dilution was used to isolate the cells, followed by a spread plate technique. It was diluted to ten-folds and 0.1 ml of the sample was taken from all the dilution was plated directly on nutrient agar and the plates were incubated at 37°C for 48 hrs. Bacterial strains were selected for further studies and the amylase producers were then sub-cultured on to nutrient agar plates in order to obtain pure isolates of bacterial species and maintained at 4°C. Isolates were identified based on various morphological and staining techniques. Only the gram-positive *Bacilli* strains were subjected to biochemical tests and were identified as per Bergey's manual of systematic bacteriology.

Primary Screening of Amylase-Producing Bacteria

Preliminary screening of amylase was done by preparing a starch agar poured into the Petri dish after sterilization. A loop full of fresh bacterial culture was picked up by a sterile inoculation loop and streaked onto the agar plate; after 24 hrs of incubation at 37°C, the plate was flooded with dilute iodine solution. Hydrolysis of starch was determined by clear zone of hydrolysis.

Amylase Production

For the preparation of production medium, a loop full of bacterial isolate was transferred in 50 ml of inoculum medium containing (g/L) starch 10, peptone 10, yeast extract 20, $MgSO_4.7H_2O$ 0.25, KH_2PO_4 0.05, $MnCl_2.4H_2O$ 0.015, $CaCl_2.2H_2O$ 0.05, and $FeSO_4.7H_2O$ 0.01. The flask was placed in incubator at 37°C for 24hrs. The supernatant was collected for amylase estimation.

Determination of α-Amylase Activity

Dinitrosalicylic acid (DNS) method was used for determining amylase activity as described by Msarah et al., (2020) using glucose has standard. A unit of amylase activity was defined as quantity of amylase needed to catalyze the release of one µmol of glucose per minute from the substrate under the test conditions was considered one unit of amylase activity. 0.5 ml of dinitrosalicylic acid, 1ml of substrate, and 1ml of enzyme sample were combined, and the mixture was incubated on a water bath for 15 minutes while cooling at room temperature. Absorbance was taken at 540nm [23].

Optimization for Production of Protease:

The *B.licheniformis* was subjected to different culture conditions to select the optimum conditions for amylase production. It was estimated at various pH, temperature, carbon and nitrogen sources.

Effect of pH

The optimum pH for amylase synthesis was investigated using a pH range of 5 to10. 100 mL of production media in 5 conical flasks were used for the analysis. 1N Hcl and 1N NaOH were used to modify the pH of the media to 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, respectively. After inoculating a 24hrs old culture and incubating it at 37°C for 48 hrs, enzyme assays were calculated.

Effect of Temperature

The production medium at pH 7.0 was inoculated with 24 hrs culture and incubated at different temperatures from 4°C, 27ºC, 37ºC, and 42ºC for 48hrs. At the end of incubation period the cell free culture filtrate was used for the enzyme assay estimation.

Effect of Carbon Source

The effect of carbon source was done based on different concentration of 2%, 4%, 6%, 8%, 10% of starch in the production medium. The production medium at pH 7.0 was inoculated with 24 hrs culture and incubated at 37°C for 48hrs. After incubation cell free culture filtrate was used for enzyme assay estimation.

Purification of the Protease

Precipitation of ammonium sulphate is frequently employed as the initial purification step and concentration technique. It's based on the salting-out process. Amylase was precipitated from crude homogenate at ammonium sulphate concentrations varying at 60%. The pellet was obtained after centrifuging the mixture at 10,000 rpm for 20 minutes at 4 °C. The pellet was suspended in a 10 ml ice-cold 0.2M phosphate buffer pH 8.0 solution. The specific activity of amylase was calculated by estimating the amylase activity and total protein content in each of the ammonium sulphate precipitated fractions. To make desalting of the proteins easier, the fraction with particular activity was dialyzed and preserved. To ensure that the resulting sample was pure, protein estimation was performed.

Protein Concentration Assay

The amount of protein was determined using the Bradford method (Kruger NJ., 1994) technique using bovine serum albumin (BSA) as standard. The results were compared with the standard curve and protein concentration was determined [24].

Immobilization of Protease

200 mg of alginate was dissolved in 10 ml Tris- Hcl buffer (0.1M pH 8.0) by heating at 30°C-40°C for 30 minutes. After all of the alginate particles had been dissolved, the liquid protease enzyme (0.5 mg/ml) was slowly stirred into the alginate solution. This mixture was stirred for 1 h for complete homogenate of enzyme and alginate. 1.4mg of Calcium chloride was dissolved in 20ml of water in a beaker and poured into petri plate. The protease-alginate mixture was added into calcium chloride by means of a syringe and was Left for solidification. Later the beads were filtered and air dried.

Characterization of the immobilized enzyme

The immobilized enzyme was used to compute the kinetic properties. DNS technique was used to determine these kinetic properties. 0.5 ml of dinitrosalicylic acid, 1 ml of substrate, and 1 ml of immobilized enzyme were combined, and the mixture was incubated on a water bath for 15 minutes while cooling at room temperature. Absorbance was taken at 540nm .

Bio-degradation of agro industrial wastes

Slight modification from An et al. (2018), was made to the established standard food waste (SFW) by The Korean Ministry of Environment Guideline (2013-179) for this study [25]. All of the foods listed in Table 1 were first ground in a blender to prepare the food waste. Before usage, the combined food ingredients were kept at 4°C. The Association of the Official Analysis Chemist's established procedures was followed to conduct the subsequent SFW analysis (AOAC). By dissolving 5 g of the sample in 25 mL of distilled water, mixing the mixture for an hour, and then centrifuging the mixture for 10 minutes at 8000 rpm, the pH of the SFW was ascertained. A portable pH meter was used to determine pH from the supernatant.

Food item	Total weight (g)	Food waste material (g)	pH of the food
Grains	100	Cooked rice	7
Vegetable	100	Onion	6.5
Fruits	100	Apple	5

Table. 1: Preparation and Formula for standard food waste

The changes in the solid contents of the SFW were calculated using the formula below:

$$\text{Solid content (\%)} = (w_2-w_0)/ (w_1-w_0) \times 100$$

Where,

w_0 = weight of the dish (g)

w_1 = weight of the sample and dish before drying (g)

w_2 = weight of the sample and dish after drying (g)

The SFW was dried in an oven to an average water content of about 80% which was determined based on the weight difference between the wet and dry samples. After this process, the SFW was used within 24 hrs.

The partly purified immobilized -amylase from *Bacillus* was utilized to treat food waste. The food waste was mixed with an aliquot of 10gm of immobilized α-amylase in a 0.85% saline solution, and the mixture was shaken at the ideal temperature of 37 °C, pH 7.0, and 200 rpm for up to 48 hours. The solid content weight and pH of the SFW were assessed for every six hours into the incubation period.

Phylogenetic Analysis

The organism's DNA was extracted using Msarah *et al*., 2020 protocol. Therefore subjected to phylogenetic analysis. The most similar bacterial species were found in the GenBank by using BLAST search (http://www.ncbi.nlm.nih.gov/). Neighbor-joining phylogenetic trees were constructed based on sequences using ClustalW [26].

Statistical analysis

All the experiments were performed in triplicate. The mean ± standard deviation (SD) was employed on the data for amylase activity, optimization at different temperatures, pH, and concentrations of carbon source, amylase purification, and bio-degradation were tested for their significance using Microsoft Excel.

Results and discussion

The selection of proper microorganisms is always crucial in the manufacture of enzymes for industrial use and their biotechnological applications. According to literature, *Bacillus* species effectively produced amylases comparing to other forms of bacteria [27-28]. Early screening of soil samples in this respect indicated the existence of 5 bacterial isolates for amylase production. During the qualitative screening, it was discovered that one of the isolates produced more amylases and had a larger zone of hydrolysis. (Fig.1). The strain was identified as *Bacillus licheniformis* by relying on Bergey's Manual of Systematic Bacteriology's key techniques (Fig. 2).

Fig.1: The amylase qualitative assay for the Bacilli-3 isolates.

According to Thebti *et al*., (2016) the activity of enzyme is revealed in the size of the clearing zone, measured by centimeters [29]. The larger the size of the clearing zone, the higher the activity produced. Further studies were carried out for quantification of extracellular amylase in liquid starch medium were the crude sample from the isolates showed higher amylase activity of 1.58±0.29 U/ml.

Biochemical test	Bacilli - 1	Bacilli – 2	Bacilli - 3	Bacilli - 4	Bacilli – 5
Indole test	-	+	-	-	-
MR test	+	+	+	+	-
VP test	+	+	+	+	-
Citrate test	+	-	+	+	+
Catalase test	+	+	+	+	+
Oxidase test	+	+	+	-	+
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
Maltose	+	+	+	+	+
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	+	+	+	+	+
Hydrogen sulphide test	-	-	-	+	-
Urea test	-	-	+	-	+

Fig. 2: Results of Biochemical test for isolated *Bacilli* species. Among the 5 isolates, Bacilli -3 was identified as *Bacillus licheniformis*

Optimization plays an important role for an enzyme to ferment the substrate. The rate at which the given substrate (starch) is broken down by amylase depends on various parameters like pH, temperature, etc. [30]. Hence, optimization was based on three parameters i.e., pH, temperature and different percentage of carbon sources. In optimum pH the maximum amylase production was seen at pH 7 ranging from 1.45±0.54 U/ml (Fig.2). This finding is consistent with the finding of Utong et al., (2006), described that at pH 6-9 range *Bacillus sphericus* was produced α-amylase [31]. In contrast, Roy et al. (2011) showed that pH 6 was the optimal pH for *Bacillus subtilis* strain AS-S01 during enzyme production [32].

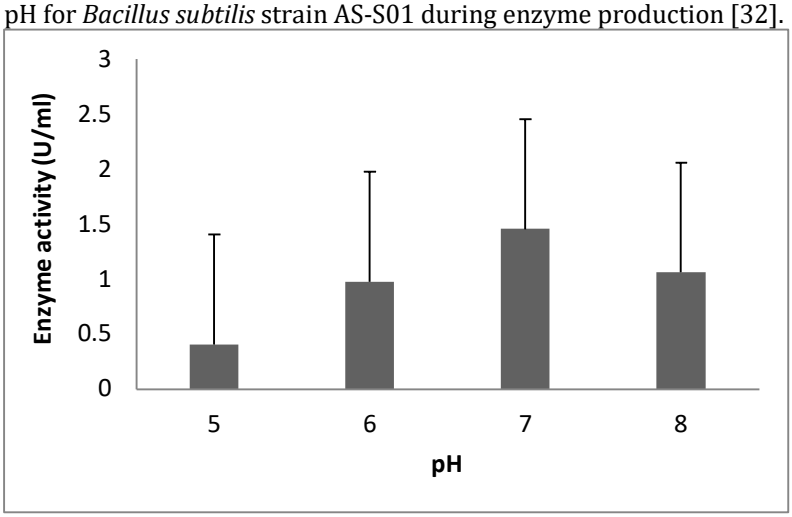

Fig. 3: Optimization of pH for amylase production

In order to be effective in industrial processes, it is necessary for an enzyme to work at residual temperature. Hence, amylase activity was determined at different temperatures and the higher activity was found be ranging from 1.19±0.06 U/ml at 37° C (Fig.4).

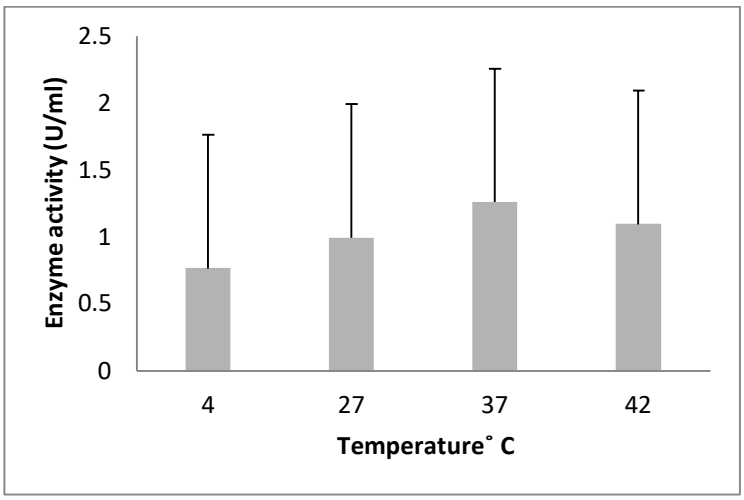

Fig. 4: Optimum temperature incubation for amylase

This is in line with Mishra et al., (2005) who reported that *B. amyloliquefaciens, B. stearothermophilus, B. subtilis,* and *B. licheniformis* are the most commonly used *Bacillus sp*. that produce amylase at temperatures 37–60°C [33]. Also, this finding is consistent with the finding of According to Alkand et al., (2018), *B. licheniformis* produced 0.7947 U/mg/ml/min of maximal α-amylase activity at a pH of 8.0. In the test to identify the percentage of carbon source the isolates were kept in varying percentage from 2 to 10 [34]. The maximum production of amylase was seen at 4% from 1.21±0.2 U/ml (Fig.5). The similar observation was seen for *B. thermooleovorans*, is reported to prefer starch, glucose, lactose, maltose and maltodextrins as carbon sources for amylase secretion [35].

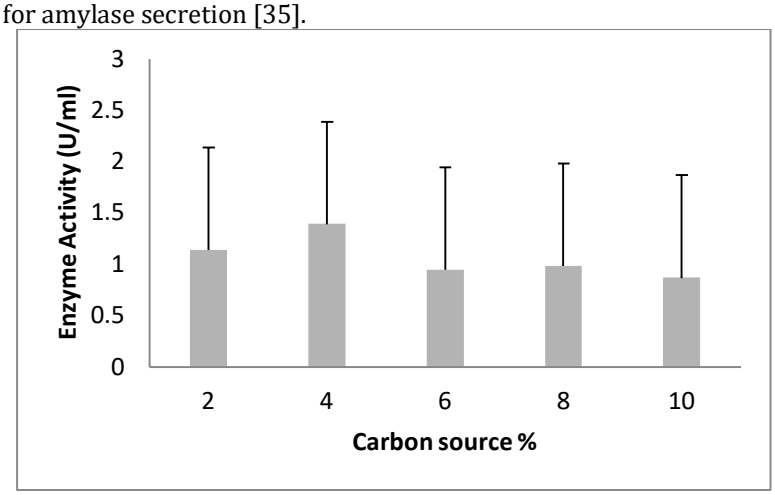

Fig. 5: Effect of carbon source on amylase activity

The 60% saturation of ammonium sulfate was used in salt precipitation step of enzyme purification, which gave a yield of 0.4±0.05% having specific activity of 3.96±0.13/mg. The acquired ammonium sulfate precipitate was introduced into dialysis bag against 0.2M phosphate buffer, and then the specific activity reached to 6.10±0.34U/mg, with purification fold 1.48±0.07and yield 0.32±0.02% (Table.2).

Sl.no	Purification	Volume (ml)	Enzyme activity (U/ml)	Total activity (U)	Protein conc.(mg/ml)	Specific Activity (U/mg)	Fold	Yield%
1	Crude	19±1.0	1.99±0.37	53.62±0.37	0.754±0.02	2.63±0.28	0.9±0.05	100±0.57
2	Ammonium sulphate (60%)	10±1.01	1.52±0.11	20±1.08	0.527±0.01	3.96±0.13	1.2±0.1	0.4±0.05
3	Dialysis	9±1.0	1.12±0.03	17.52±0.266	0.276±0.01	6.10±0.34	1.48±0.07	0.32±0.02

Table. 2: Partial purification steps of α-amylase from *B. licheniformis*

Immobilization of amylase on to appropriate support materials plays an essential role in various fields of technology including the food and detergent industries. As a result of its uses in bio catalysis, enhancement of amylase immobilization has received great praise [36]. In this research, amylase was encapsulated in sodium alginate and amylase assay was determined. The results demonstrated that the amylase enzyme after immobilization in alginate beads exhibits reasonable stability and good activity (1.48±0.11U/ml). In contrast to previous research, the *P. chungwhensis* amylase used in the experiment was immobilized by being trapped inside calcium alginate beads and then incubated with the substrate solution. It was shown that when the immobilized enzyme and substrate solution interacted longer, the amylase activity increased. The maximal amylase activity, as 90.3 U mL^{-1}, was discovered after 180 min of incubation, resulting in 69.6% immobilization efficiency [37].

Furthermore, to the earlier study by Mamo et al., (1997), immobilized cell form demonstrated an increase in amylase activity by 2 UmL-1 compared to the free cell [38]. This demonstrated conclusively that direct contact between a biocatalyst and a substrate boosts amylase activity. Thus, this study suggesting a potential application for this immobilized enzyme in the food, detergent, and textile industries. Food wastes naturally include a variety of organic materials that can act as carbon sources for a variety of microbes. Upon utilization of these carbon sources by the microbes, the quantity of food wastes can be effectively reduced. Numerous studies have been conducted on the use of aerobic and anaerobic fermentation techniques to remediate food waste using microorganisms [39].

Among the thermophilic and mesophilic microbes found in food waste, *Stearothermophilus spp*, *Pseudomonas spp*., and *Bacillus spp* are also included [25]. The high growth rate of *B. subtilis* and *B. licheniformis*, as well as their ability to release several hydrolytic enzymes has made them as attractive industrial bacilli [40]. Early studies by Seo et al., (2013), Parrado et al., (2014), and Sewalt et al., (2018) reported *B. subtilis* and *B. licheniformis* strains produced multiple enzymes that can mediate several actions, can degrade different substrates, and flourish under various environments [41- 43]. Hence, to our knowledge studies on using purified amylase from mesophilic *Bacillus* for the application of food waste treatment, is still novel.

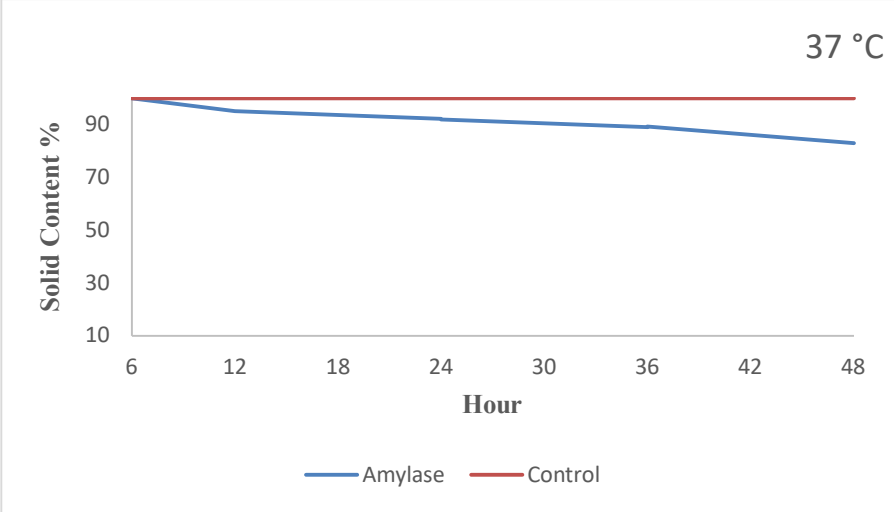

Fig. 6: The solid content of SFW treated with immobilized α-amylase extracted from *B. licheniformis* at 37 °C.

Amylase from *Bacillus* isolated from domestic waste has been employed in the preponderance of research as a biodegradable substance [44-45]. Kitchen wastes mostly consist of starchy materials (similar to the agricultural waste in this study); they are also a rich source of starch degrading microorganisms. Utilizing microorganisms to transform waste into useful products might significantly reduce the amount of waste dumped in landfills [46]. The cellulolytic and proteolytic activities of *B. subtilis* and *B. licheniformis* have both exhibited strong ability to breakdown food wastes [25]. *Bacillus* species may use keratinous wastes as their only source of carbon and nitrogen for growth [47]. Keratinase enzymes are a particular class of protease with the ability to biodegrade substrates [48]. As a result, the majority of *Bacillus* spp. produced enzymes is still active and will be in responsible of reducing the solid contents of food wastes. The food wastes treated with α-amylase were incubated at different time intervals from 6hrs to 48hrs to obtain a maximum decrease in dry weight (Fig.6). *B. licheniformis* in its immobilized form demonstrated the most weight loss with the amylase. The initial pH was 7.5 and at the end of the experiment it reduced to 5.9. The α-amylase at 37 °C resulted in a decrease in weight of about by 83% ± 0.03 of solid content.

During phylogenetic analysis the sequence was compared using the Basic Local Alignment Search Tool, which was used on multiple sequence alignments to create a phylogenic tree with branch length of 16S r-RNA sequence using CLUSTALW (BLAST). Using MEGA 7.0 software, a phylogenetic tree with maximum likelihood (ML) was created to determine the connection between our isolates and other reference strains. Phylogenetic analysis of our isolate, 16Sr ribosomal RNA gene sequences revealed that the amylase producing bacteria was confirmed as *Bacillus licheniformis.* Results revealed that among *Bacillus aerius, Bacillus paralicheniformis, Bacillus piscis, Bacillus glycinifermentans* and *Bacillus haynesii*, only *Bacillus aerius* showed closely relationship with *B. licheniformis* having common ancestor (Fig. 7).

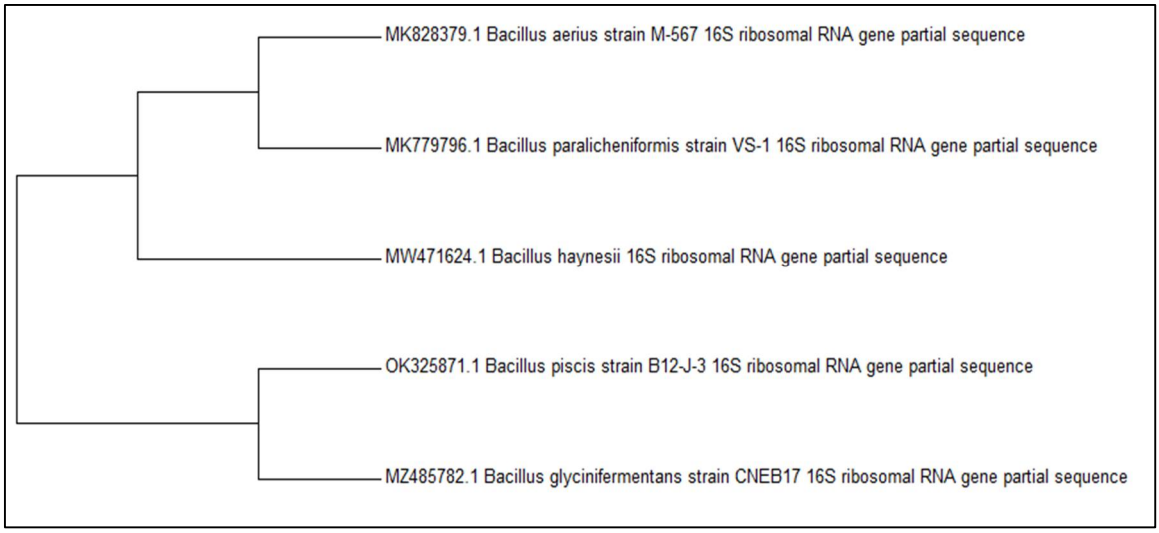

Fig. 7: Result of Phylogenetic analysis for *Bacillus licheniformis*, constructed using Mega 7

CONCLUSIONS

From the present study, it is concluded that among isolated bacteria from soil with amylolytic activity, *B. licheniformis* was capable to have the maximum microbial growth and highest α-amylase enzyme production at pH 7.0 and the optimum temperature was 37°C. The amylase was purified by ammonium sulphate precipitation and dialysis. The purified amylases were immobilized using sodium alginate and amylase activity was determined. They were able to grow at 37°C temperatures in an SFW mixture, causing in substantial reduction of the food materials. The findings showed that the strain displayed novel properties to favor the degradation of food waste at a faster rate by α-amylase.

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