

## IMMOBILIZATION OF AMYLASE PRODUCING BACTERIA (*BACILLUS SUBTILIS*) FROM SOIL OF WESTERN HIMALAYAN REGION SOLAN, HIMACHAL PRADESH, INDIA

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### ABSTRACT

**Objective:** The paper aimed to immobilize amylase producing bacterial strain on a suitable matrix and characterization of its physicochemical properties so that much amount of amylase could be produced to be applied in different industries.

**Methods:** Bacterial colonies were sub-cultured from samples collected from soil in freshly prepared dishes containing starch agar by dot method using sterile inoculating needles from which five different bacteria belonged to genus *Bacillus* were isolated and assigned as A1, A2, A3, A4, and A5.

**Results:** It was found that A1 displayed the highest enzyme activity of 17.89 IU/ml with enzyme assay of 0.83 mg/ml and the bacterium was identified to be *Bacillus subtilis*. A5 displayed 10.13 IU/ml with protein contents of 0.11 mg/ml indicated that A1 possess the highest enzyme activities which were categorized under *Bacillus* and protein contents and A5 showed less amount of enzyme activities and protein contents as compared to other.

**Conclusion:** The bacteria which were produced much amount of enzyme activities identified as *Bacillus subtilis* and recommended and have been recommended to be cultured for the production of amylase enzyme.

**Keywords:** Amylase, Immobilize, Bacteria, Bacillus, Culture.

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### INTRODUCTION

Amylase is an enzyme that hydrolyses starch into dimeric compounds by hydrolysis reaction then into the smallest glucose [1]. Amylase is a very prevalent enzyme produced biologically by plants, animals, humans, and microorganisms [2]. A large portion of the enzyme market share is owned by amylase [3]. A wide range of industries such as food industries, garments, textiles, beverage, and medicinal uses amylase to manufacture their products [4]. Extraction of this huge quantity of amylase directly from nature is not feasible and hence various methods are being constantly established to develop the mass production of commercial amylase [5].

Bacteria are the most preferred organism to produce different enzymes among which the *Bacillus* spp. are well recognized as amylase and protease producers [6]. Some of these include *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus*, *Bacillus cereus*, *Bacillus polymyxa*, *Bacillus coagulans*, and *Lactobacillus plantarum* [2,5,7]. Other bacteria such as *Clostridium thermosulphurogenes*, *Proteus*, and *Pseudomonas* spp. are also acknowledged as amylase producers [7]. Among the various types of amylase, the microbial amylase meets the industrial demands because it can be produced in large quantities [8]. Especially, enzymes from fungal and bacterial sources have dominated applications in industrial sectors [9]. Quite a large variety of microorganisms have been identified and chosen as the source of amylase production because of the availability and simplicity of how they yield amylase. Each strain of bacteria requires specific growth conditions and nutrients to produce amylase. Soil is a primary source of these bacteria which can be isolated and commercially grown in large numbers to produce a vast amount of amylase [10]. The bacteria need optimization and determination of pH and temperature for the production of an enzyme for proper application in the industry [11].

The use of immobilized cells offers several advantages over free cells, such as the relative ease of product separation, re-use of biocatalysts, prevention of washout, reduced risk of contamination, and operational stability. Furthermore, using the entrapment technique, dense cell culture can be established that leading to improve productivity using proper immobilization methods [12,13].

The objective of the present study was to immobilize bacterial strain producing amylase on a suitable matrix and to characterize its physicochemical properties of the conditions in which a huge amount of enzymes could be produced. The study may help us in isolation of certain microorganisms specifically, amylase-producing bacterial strains immobilizing them on a suitable matrix which can further be used in wastewater treatment, bioreactors, food, textile, detergent, and paper industry.

### METHODS

#### Collection of samples

Soil samples were collected from Krol hill, Solan district of Himachal Pradesh with the help of a sterile spatula. The samples were transferred into sterile plastic bags and stored in a refrigerator at 4°C for further processing.

#### Isolation of amylase producing bacterial strain

Isolation of amylase producing bacteria was performed by serial dilution and spread plate method. One gram of soil samples serially diluted in sterilized distilled water, and a volume of 0.1 ml of appropriate dilution transferred aseptically to nutrient agar plates. The specimen was spread uniformly using a sterilized glass spreader and then incubated at 37°C for 24 h. The bacterial isolation further subcultured to obtain a pure culture [5].

### Screening of amylase producing bacteria

Colonies of bacterial subcultured in freshly prepared dishes containing starch agar by dot method using sterile inoculating needles. The plates were then incubated at 37°C for 48 h in the incubator. After incubation, the plate flooded with Gram's iodine. Any formation of a clear zone around the colonies were observed and the diameters were measured using a ruler. The isolates observed with the largest clear zones around them were selected [10].

### Identification of amylase producing bacteria

#### Cultural characterization

The morphology of the colony isolate was observed, and the results were noted concerning color, shape, size, and nature of colony pigmentation [11].

#### Microscopic observation

The bacterial isolates were Gram-stained and observed under a high power magnifying lens in a light microscope. Endospore staining and motility test were performed to observe the morphology and motility of the cells [11].

#### Biochemical characterization

The bacterial isolates were characterized biochemically by indole test, methyl red test, Voges-Proskauer (VP) test, Simmons citrate test, catalase test, urease test, nitrate reduction test, H<sub>2</sub>S production, coagulase test, and carbohydrate fermentation [11].

### Quantitative determination of amylase activity

#### Preparation of seed culture

Fifty milliliters of seed culture were prepared by inoculating a loopful of pure culture in the media. The flask was incubated for 24 h with continuous shaking (130 rpm) at 37°C (Orbitex, Scigenics Biotech).

#### Enzyme production

Two milliliters of seed culture were incubated in 50 ml of production media (beef extract 0.25%, peptone 0.15%, and starch 1%) and grown for 48 h in a shaker incubator at temperature 37°C and 130 rpm. The production medium centrifuged at 5000 rpm for 10 min. The supernatant was collected and stored in the refrigerator for further use. It was performed following by Ashwini *et al.* [14].

#### Enzyme assay for amylase enzyme

Amylase activity was determined using [15] method. One milliliter of enzyme extract was added to 1 ml of substrate solution (containing 1% soluble starch added in 0.2 M phosphate buffer pH 6.9) and incubated at 30°C for 30 min in a water bath. A blank was set up consisting of 1 ml of enzyme extract that has been boiled for 20 min, and then the starch solution was added to it. Two-milliliter DNS reagent was added to each tube to stop the reaction, and tubes were kept in a boiling water bath for 5 min. Tubes were cooled at room temperature, and the absorbance was taken at 540 nm by a spectrophotometer. Enzyme activity was expressed in units (1 unit/ml=amount of enzyme which releases 1 μ mole glucose under the assay condition).

#### Enzyme assay

To determine the specific enzyme activity of the selected isolates, the Folin-Lowry method for total protein estimation was used [16]. To each test tube, a 0.2 ml crude enzyme (supernatant) was added. To that, 2 ml alkaline copper sulfate (reagent C) was added which was made using reagent A and reagent B before use. The test tubes were incubated in the dark for 10 min. After incubation, 200 μl of Lowry reagent was added to the test tubes. A further incubation was done for 30 min in the dark. The absorbance of the solutions was then measured in optical density using a spectrophotometer at 650 nm. The readings were compared to a prepared blank solution. The readings were used to find out the unknown protein concentration from the

protein standard curve. The specific enzyme activity was measured using the following formula.

$$\text{Specific activity} \left( \frac{\text{U}}{\text{mg}} \right) = \frac{\text{Enzyme activity (U/ml)}}{\text{Extracellular protein concentration (mg/ml)}}$$

### Standardization of calcium alginate beads by physical entrapment and immobilization of amylase producing bacterial strain

#### Biomass generation

A pure isolated colony of bacterial strain on the starch agar plate inoculated in starch broth in 1 L flask. The biomass was generated by incubating this flask at 37°C in the shaker at 120 rpm under aerobic conditions. After 24 h of incubation, the cells were harvested at 6000 rpm for 7 min at 4°C (Eppendorf Centrifuge 5430 R). The supernatant was discarded, and biomass was washed twice with sterile phosphate-buffered saline (PBS), pH 7.0 at 6000 rpm for 5 min at 4°C. Finally, the biomass was suspended in 1 ml of sterile PBS [13].

### Standardization of sodium alginate concentration for immobilization of bacterial strain

The generated biomass was adjusted to a concentration of 1 g/L and cell suspension was used as inoculum for the preparation of calcium alginate beads with the size of the inoculum 10%, 20%, 30%, 40%, and 50% in various concentrations of sodium alginate in sterile PBS under aseptic conditions to a final volume of 10 ml. This sodium alginate containing bacterial cells was aseptically extruded dropwise through a syringe into a gently stirred ice-cold sterile 1 M CaCl<sub>2</sub> solution. The beads were kept in CaCl<sub>2</sub> solution at room temperature to allow them to harden for 20 min. The beads were finally washed with distilled water [13].

### Analysis of calcium alginate beads containing amylase producing microorganism

#### Leaching

The total beads were prepared in various reaction mixtures and taken in 50 ml of aliquots were withdrawn from the flask after every hour, and absorbance of buffered starch was taken at 540 nm against buffered starch as blank.

#### Stability

The hardness of the beads was tested by taking the entire beads removed from the flask, and the stability of the beads was checked by pressing the beads between the thumb and forefinger. The beads which did not break over 15 days were selected for further studies [17].

#### Culture viability

The beads withdrawn from the flask were crushed in a sterile mortar-pestle in 1 ml sterile PBS aseptically. The cell suspension was streaked on nutrient agar plates and the plates were incubated at 37°C for 24 h. Those beads which showed bacterial growth up to 10 days in the immobilized matrix were selected for further studies [17,18].

### Determination of enzyme activity in immobilized cells

#### Glucose standard curve by DNS method

A standard stock solution of glucose (150 μg/ml) was prepared in distilled water. A standard curve of glucose with concentration ranging from 0 μg/ml to 150 μg/ml was prepared by taking 3 ml of glucose solution and 1 ml of DNS in boiling tubes. The tubes were kept in a boiling water bath for 5 min. After cooling the tubes, absorption was taken at 540 nm and the standard curve of glucose was plotted between glucose concentration and absorbance [19].

#### Determination of enzyme activity

Beads prepared were incubated in 50 ml of 1% buffered starch in 250 ml of the conical flask. After every hour, 3 ml of the starch solution

was aliquoted and the reaction was stopped by adding 200 µg of 0.1 N NaOH. The enzyme activity in each aliquot was determined in terms of international units. One unit was defined as the amount of the enzyme present in 10 ml of calcium alginate beads which showed maximum stability, culture viability, and enzyme activity with minimum leaching were selected for further studies [20].

### Optimization of physiochemical parameters

#### Temperature optimization

The temperature of the medium in which the inoculums performed well determined by the selected beads which were incubated at a different temperature over a range of 37°C-40°C in 20 ml of 1% buffered starch for 5 h. After every hour, 3 ml of aliquot was withdrawn and the enzyme activity was determined.

#### pH optimization

The selected beads were incubated in 20 ml of 1% buffered starch maintained at different pH (over a range of 6-8) with a variation of 0.2 for 5 h and optimized temperature. Three milliliter aliquots were withdrawn after 5 h and enzyme activity was calculated [20].

## RESULTS AND DISCUSSION

### Isolation and screening of amylase producing bacteria

Five bacterial strains were isolated from the soil sample. All five isolates were found to hydrolyze the starch. The amylolytic activity of these isolates and their zone of hydrolysis were observed in Table 1 and Fig. 1. Strain A1 shows the maximum zone of hydrolysis around the colony on a starch-containing medium. The results were similar with [17,21-24]. It showed a similar result with an experiment performed on samples extracted from yam peel dumpsite by Kar *et al.* [25]. Again the experiment extracted from Cassava tubers came up with similar results [26]. Mishra and Behera [23] also reported amylase activity with a clear white zone around the colony of *Bacillus*. Ahmed *et al.* [27] also reported that *B. acidocaldarius* gave the largest zone of starch hydrolysis and it was the potent strain for  $\alpha$ -amylase production. As it shown, 0.83 mg/ml of protein (enzyme) was measured as compared to other strain A2 (0.61 mg/ml), A3 (0.29 mg/ml), A4 (0.73 mg/ml), and A5 (0.11 mg/ml). Strain A1 and A4 shown greater production of enzyme than the other three and strain A1 was greater than strain A4 bacteria and this strain was categorized into *Bacillus* species (Table 1).

Fig. 1 indicated that the zone of hydrolysis for the A1 strain of bacteria was higher than other strains. This strain of bacteria produced much amount of enzymes that can hydrolyze starch. Data indicated in Table 2 showed the morphological test, character, and remarks of the isolated bacterium which is very important to identify the specific bacterium that applied to produce much amount of amylase enzyme. According to



Fig. 1: Zone of hydrolysis shown by A1 strain on starch agar plate

the information stated in Table 3, the bacteria which were immobilized to produce much amount of amylase enzyme classified as *Bacillus* spp.

### Gram's staining

The selected bacteria were observed to be Gram's positive rods when visualized under the microscope after Gram's staining (Fig. 2).

### Spore staining

After the Gram's staining spore, staining was done and it was endospore forming bacteria (Fig. 3).

### Biochemical tests

A biochemical test was performed after the Gram's staining. A different biochemical test has been made which confirmed that the isolated bacterium was *Bacillus*. Positive biochemical tests observed with biochemical tests such as catalase, coagulase, VP, citrate, and other biochemical test were resulted such as urease, indole, triple sugar iron, mannitol, sucrose, and magnetic resonance showed negative results. The presence of sucrose and absence of glucose also another indication of amylase enzyme that was produced from bacterium as indicated in Table 2 and Fig. 4. The characterization of all these biochemical tests in Table 2 was confirmed that the genus of bacterium immobilized was *Bacillus*.

Table 1: Zones of hydrolysis show enzyme activity and protein content for different bacterial isolates

Isolation code	Zone of hydrolysis	Enzyme activity (IU/ml)	Protein Content (mg/ml)
A1	++	17.89	0.83
A2	+	13.33	0.61
A3	+	12.62	0.29
A4	++	14.60	0.73
A5	+	10.13	0.11

++: Maximum zone, +: Minimum zone

Table 2: Biochemical test result + and - positive and negative result

Biochemical tests test results	
Catalase	+
Urease	-
Coagulase	+
Indole	-
Triple sugar iron	-
Magnetic resonance	-
Voges-Proskauer	+
Citrate	+
Glucose	A
Lactose	-
Sucrose	+
Mannitol	-

Table 3: Identification of selected amylase producing bacteria morphological characters

Morphological tests	Character	Remarks
Colony characteristics	Configuration	Round
	Margin	Undulated
	Elevation	Raised
	Surface	Rough
	Density	Opaque
Gram's staining	Pigments	None
	Shape	Rod like (bacilli)
Spore formation	Color	Violet (Gram +)
	Endospore	Present
Motility	Position	Central
	Shape	Oval
		Positive (+)

### Genetic identification of bacterial isolate

After complete identification by Gram's staining and biochemical test, it was observed that the identified culture was *Bacillus* sp. Furthermore, we have identified the bacteria using biochemical test and morphological criteria, the bacteria's (A1) the genetic identity have been analyzed to confirm that the isolated bacteria was *B. subtilis*. 16S rDNA sequencing is one of the most common techniques used to study the taxonomy and phylogeny of bacteria. Using Quick-DNA fungal/bacteria Miniprep Kit (Zymo Research, India), the isolated bacterial genome has been extracted and

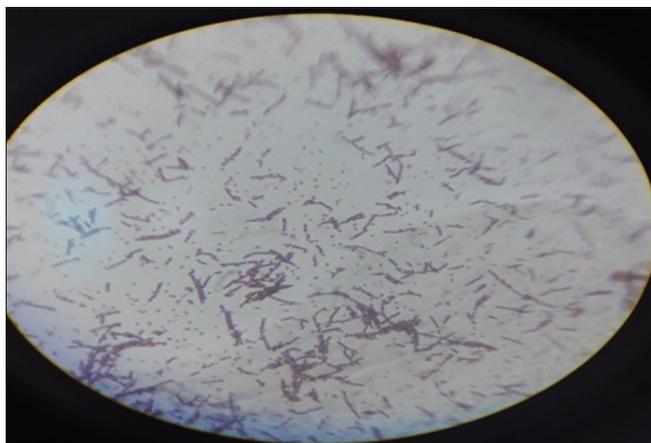


Fig. 2: Appearance of Gram's positive rods

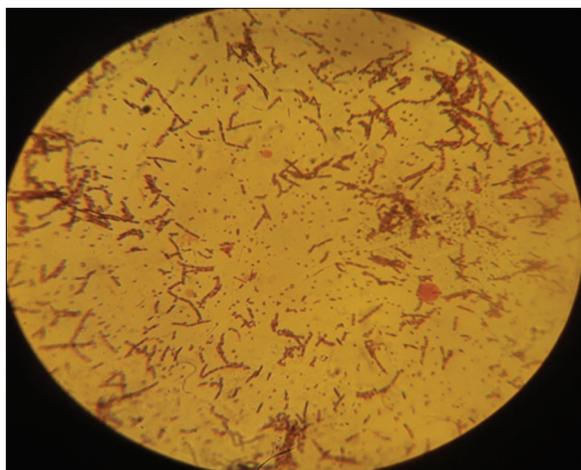


Fig. 3: The spores are stained green whereas the bacterial cells are stained red

then purified from bacterial strains was carried out as per the manual. The 16S rDNA gene has been amplified using universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5'GGTTACCTTGTACGACTT-3'). The amplicons were purified using QIAquick Gel extraction kit (Qiagen, India). DNA sequencing was performed and the nucleotide sequences were compared against GenBank database using the basic local alignment search tools (BLAST) software provided online by the National Center for Biotechnology Information (NCBI) algorithm and deposited in the NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>). The results of sequencing revealed that bacterial strain isolated from soil with higher enzyme activities was *B. subtilis*.

### Standardization of sodium alginate concentrations and percent inoculums for immobilization of bacterial strain

Various concentrations of sodium alginate were used to achieve maximum immobilization efficiency as the degree of cross-linking directly affects pore size resulting in easy availability of nutrients to the cell and cell leakage from the entrapped matrix. Among various concentrations of alginate used, 3% concentration was found to be suitable for amylase production by *Bacillus* sp. [28] reported that 8% concentration of alginate was found to be suitable for  $\beta$ -amylase production by *B. subtilis*.

It was observed that with a lower concentration of sodium alginate which was 1% and 2% the beads formed were not spherical and therefore they were discarded; 3% sodium alginate concentration gave stable beads is shown in further table and plate and those beads were checked for their enzyme activity with 10%, 20%, 30%, 40%, 50%, and 60% inoculums size. It has been reported that alginate and  $\text{CaCl}_2$  concentrations used in cell entrapment range 2–5%. The capsules formed from sodium alginate solutions lower than 0.5% (w/v) had gelled poorly, produced sticky surfaces, and were fragile and difficult to handle [20].

As it was indicated in Table 4, as the percentage of inoculums increased the rate at which enzyme catalyzed the substrate decreased with extended time of incubation. At 5 h of inoculation time, the rate of enzyme activities showed positive result in all inoculums of

Table 4: Analysis of calcium alginate beads (prepared with 3% sodium alginate solution); cell loading 10

Incubation time (h)	Leaching ( $A_{540}$ )	Bead stability	Culture viability
5 h	0.01	Rigid	+
24 h	0.01	Rigid	+
10 days	N.D	Rigid	+
15 days	N.D	Rigid	-

N.D: Not determined



Fig. 4: Biochemical tests of isolate A1

10, 20, 30, 40, 50, and 60%. At 24 h of inoculation time, the rate of enzyme activities showed positive result in 10, 20, 30, and 40, but showed negative result 50% and 60% of inoculums. This was because resource depletion and wastes discharged caused the enzyme activities to be negative result. Again at 10 days of inoculation time, the rate of enzyme activities showed positive result only in 10% inoculums, but showed negative results in 20%, 30%, 40%, 50%, and 60% of inoculums. This was because as the population of bacterium increased it caused the resource depletion and enzyme become devoid of substrate to catalyze and subsequently stop the activities. Besides, wastes discharged in the course of extended inoculation time caused the enzyme activities to be negative result (Table 5 and Fig. 5).

Fig. 5 revealed that the inoculums size affected the enzyme activities which produced from bacteria. This indicated that as the population of bacteria increased from the load of much amount of inoculums, the carrying capacity of the medium in which it had been cultured reached and could not support the growth and survival of inoculated bacteria. At 10% of inoculums size the enzyme activities was about 16.2 IU/ml and as the inoculums size increased the enzyme activities of inoculums subsequently decreased and therefore, at 60% inoculums size the enzyme activities observed was about 2.3 IU/ml.

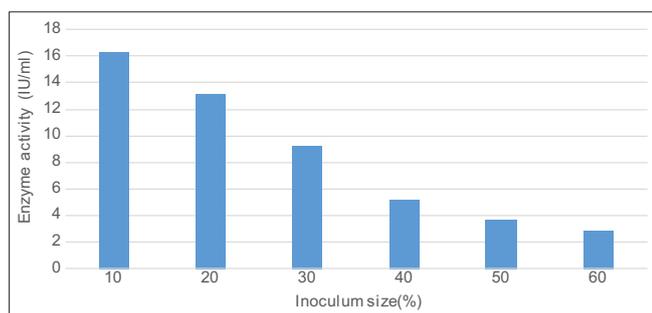


Fig. 5: Effect of inoculums size on amylase activity in immobilized cells

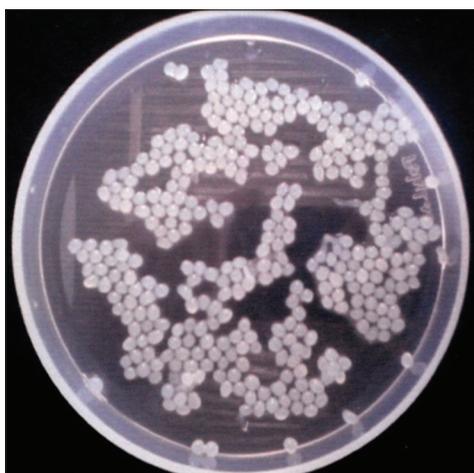


Fig. 6: Calcium alginate beads prepared from 3% sodium alginate solution

### Leaching

As depicted in Table 5, calcium alginate beads immobilized with 10% of inoculums are effective for immobilization of bacterial strain in calcium alginate beads (Fig. 6). About 10% cell loading was, therefore, selected for further studies. As the biomass concentration in the gel beads increased, the cell leakage into the fermentation medium also increased correspondingly. This could be attributed to the fermentation medium also increased; the nutrient/cell ratio decreased which might become limiting [29]. Konsoula and Liakopoulou-Kyriakides [20] reported that an increase in the biopolymer concentration from 2% to 5% (w/v) improved the durability of the capsules and reduced cell leakage by 7%.

### Stability

Table 5 shows that the beads with 10% inoculums were stable up to 15 days, whereas 20% and 30% inoculums were stable up to 24 h. This indicates that the higher percent inoculums did not allow the stable cross-linking of calcium alginate in bead formation. Therefore, the bead loses its integrity with a short time spent of 5 h.

### Culture viability

About 10% inoculums were selected for the further studies as the culture was viable up to 10 days, as shown in Table 5.

### Determination of enzyme activity in immobilized cells

The amylase activity was found to be the maximum with 10% inoculums throughout 5 h beginning with 10.639 IU/ml in 1<sup>st</sup> h and increasing gradually up to 16.460 IU/ml in the 5<sup>th</sup> h. Kumar *et al.* [30] reported that the amylase production by immobilized cells reached 12.32 U/ml by 16 h, while 11.96 U/ml activity was reached by 24 h with free cells.

### Optimization of physicochemical parameters

After we have identified the bacterial strain which produced much amount enzyme, then we performed the optimum condition that should be full filled. The bacterial strain which able to produce much amount of amylase was labeled as A1 belonged to *Bacillus subtilis* (Table 3). As it has been indicated in Fig. 7, the enzyme activity this bacterial strain was greatly enhanced in 5 h with further increase in incubation time not much of enzyme activity was seen for the temperature, that is, 37°C, 38°C, 39°C, and 40°C. The optimum temperature for amylase activity was found to be 38°C (22.5 IU/ml) and with the increase in temperature amylase activity decreased. It has been observed previously by Kumar *et al.* [18] that  $\alpha$ -amylase entrapped in calcium alginate gives an enzyme activity of 17.64 IU/ml units at 37°C.

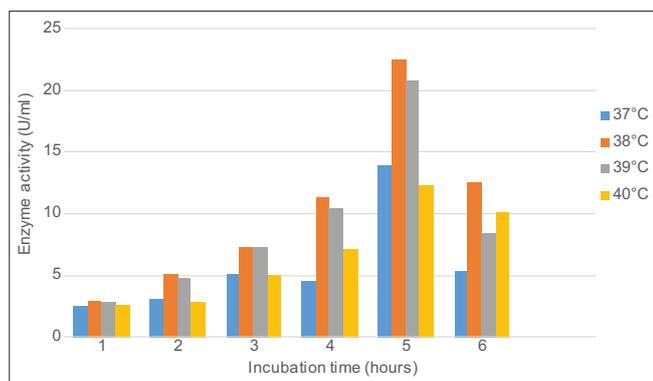
Fig. 7 indicated that at 1 h of inoculums time maximum enzyme activities observed were about 3 IU/ml. At 2 h of incubation time, the maximum enzyme activities observed were 5 IU/ml, and at 3 h of inoculums time the maximum of around 7 IU/ml. The enzyme activities were about 12 IU/ml at 4 h of inoculation time. The maximum enzyme production was observed at 5 h of inoculums time. Overall enzyme activities (23 IU/ml) were observed at 5 h of inoculation time but the enzyme activities were decline again at 6 h of inoculation time indicating that as the inoculation time extended the enzyme activities decreased. The extension of inoculation time resulted in depletion of resources and increased the metabolic wastes in the medium which could hinder the metabolic process enzyme secretion.

The result of Fig. 8 indicated that at low pH and higher pH did not suit the bacteria for the production of massive amounts of amylase enzyme and its activities. It has been reported previously by Dhanasekaran [19] that the optimum pH for amylase production by immobilized *Bacillus*

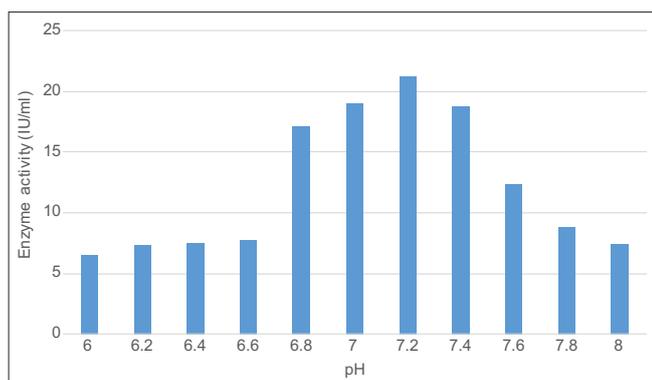
Table 5: Calcium alginate bead stability (with 3% sodium alginate solution) with varying cell loading

Incubation time	10% inoculums	20% inoculums	30% inoculums	40% inoculums	50% inoculums	60% inoculums
5 h	+	+	+	+	-	-
24 h	+	+	+	+	-	-
10 days	+	-	-	-	-	-

+denotes that the beads were maintaining their rigidity



**Fig. 7: Effect of temperature on amylase activity in immobilized cells**



**Fig. 8: Effect of pH on amylase activity in immobilized cells**

sp. was in the range of 6.5–7.5. The result of this study also showed that between the ranges of pH 6.8–7.4 the enzyme activities displayed by the immobilized bacteria were higher with the highest enzyme activities at pH 7.2 (21.81 IU/ml).

#### CONCLUSION AND RECOMMENDATION

The strain A1 belonging to *B. subtilis* was effectively entrapped in calcium alginate beads with 3% sodium alginate and 1 M calcium chloride. About 10% cell loading was found to be optimum the maximized  $\alpha$ -amylase production (17.86 IU/ml) without any observable cell leaching for 24 h and the beads were stable up to 15 days and culture viability up to 10 days. The optimum temperature of immobilized cells for  $\alpha$ -amylase production was found to be 38°C (22.5 IU/ml) and the optimum pH was found to be 7.2.

Since the soil sample from which the bacteria were screened and immobilized from the hilly area of Himachal Pradesh, a concerned industrial sector able to screen and immobilize the bacteria which produce much amount of amylase enzyme for application to form quality products using this enzyme.  $\alpha$ -amylase is of great significance with applications in food, baking, brewing, fermentation, detergent, textile designing, and paper industries. The immobilized microorganism technology offers a multitude of advantages in wastewater treatment. Immobilized cells have also been used in bioreactors, and the production of useful compounds such as amino acids, organic acids, antibiotics, steroids, and enzymes. Therefore, the application of the finding this paper could enhance the production of many amounts of this enzyme to be applied in a different sector

#### AUTHORS' CONTRIBUTIONS

Arun Kumar and Kasahun Gudeta had been performed lab analysis, Prof. JM Julka supervised the performance but other author contributed to finalize the manuscript.

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#### CONFLICTS OF INTEREST

None.

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