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Screening, Isolation, Identification, Whole Cell Immobilization and Optimization of *Bacillus amyloliquifaciens* for Production of Amylase.

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Abstract

Amylases have several commercial applications. Microbial amylases are widely used in industries. Present study focuses on screening, isolation, identification, whole cell immobilization and optimization of *Bacillus amyloliquifaciens* isolated from soil. 20 typical colonies of *Bacillus* spp were isolated on starch agar plate. When the plates were flooded with dilute iodine solution and observed for clear zone of hydrolysis, the maximum zone diameter was 18 mm given by B6 isolate. The isolate was identified by VITEK-2 automated system as *Bacillus amyloliquifaciens*. It was subjected to whole cell immobilization using cell entrapment by calcium alginate. Activity of amylase produced by free cells and immobilized cells was estimated using DNSA method. Crude preparation of Amylase produced by free cells showed activity of 2.78µM/ml/min. Whole cell immobilization of cells using calcium alginate resulted into 2 times improvement of enzyme activity which was 5.53µM/ml/min indicating enhanced production of enzyme. Study of optimization of parameters for enzyme produced by immobilized cells indicated pH 6.5, temperature 25^0C and substrate concentration 1% as the most suitable parameters for getting maximum enzyme activity by immobilized *Bacillus amyloliquifaciens*.

Keywords: Amylase, *Bacillusa myloliquifaciens*, Calcium alginate, DNSA assay,, Whole cell immobilization, Optimization.

1. Introduction

Extracellular digestion of polymers like starch is essential if it is to be used as nutrient by micro-organisms. Several micro-organisms produce amylases extracellularly. Two major classes of microbial amylases are α-amylase and β-amylase.α-amylases (1,4-α-D-glucan glucohydrolase) are endoenzymes, randomly cleaving α-1,4 glucosidic linkages in amylose portion of starch and produce mixture of dextrins, glucose and maltose. β-amylases are exoenzymes which act on amylose and amylopectin of starch systematically and cleave alternate α-1,4 glucosidic linkages from non-reducing end and produce a mixture of dextrins and maltose (Keharom *et al*,2016; Nelson *et al*, 2000). Both these enzymes do not cleave α-1,6 glucosidic linkages present in starch.

Amylase has wide commercial applications. It is used for preparation of sugar syrups from starch which can be used in chocolate making. It is used in pharmaceutical industry as a digestive aid and in textile industry for desizing of fabric (Saxena *et al*, 2011, Whitaker *et al*, 2003).

Plants, animals and micro-organisms produce amylase. Microbial amylases have great potential at industrial scale. Among micro-organisms, *Bacillus* spp. are most preferred due to their simple nutritional requirements, rapid growth and production of several extracellular enzymes (Pandey *et al*, 2000).

For screening of amylase producing bacteria isolated from natural sources, most commonly, researchers have used starch agar and dilute iodine solution (Clark *et al*, 1958; Padhiar and Kommu, 2016).

Conventional methods used for identification of bacteria include study of morphological, cultural, biochemical characteristics and identification using Bergy's manual of determinative bacteriology (Banupriya and Gowrie, 2012). However, this method is time consuming and not accurate. VITEK 2 is a fully automated system that performs bacterial identification based on results of suitable biochemical tests and can give up to 99% guaranteed identification of bacteria. It is faster to complete and gives more reliable results in the identification of bacteria. Researchers have isolated protease producing bacteria from food processing industries and identified them using VITEK-2 automated system and got 85.5% probability of accurate identification. They have mentioned several advantages of this system over conventional methods used for identification of bacteria (Sony and Potty, 2017).

Whole cell immobilization is a preferred technology to enzyme immobilization. Purification of enzyme and then its immobilization is time consuming and costly affair (Elakkia *et al*, 2016).Immobilization of cells has several advantages. They show greater metabolic activity, less sensitivity to adverse environmental conditions (Ramkrishnan *et al*, 1999). Another advantage of this techniques is, it helps retain high cell concentrations within the bioreactor. Moreover, immobilized microorganisms can be used several times without significant loss of activity, proving to be cost effective (Devi and Sridhar, 2000).

Two widely used methods for determination of amylase activity are the Nelson-Somogyi (NS) and3, 5-dinitrosalicylic acid (DNS) assays for reducing sugars (Gusakov *et al*, 2011).DNS method is more commonly used for its simplicity and accuracy. In this method amylase activity is measured in terms of µM of glucose or maltose, the product produced by amylase by a unit volume of enzyme in unit time. Glucose and maltose are reducing sugars which reduce DNS to 3 –amino,5 nitro salicylic acid which is dark red in colour and estimated spectrophotometrically (Plummer 2000).

Keharom *et al* in 2016 have carried out optimization study of purified α-amylase obtained from *B. subtilis* using surface response methodology by DNS method. 1% starch concentration, 7.3 pH and 39 ^{0}C temperature were found to be the most optimum parameters for the enzyme.

Elakkiya *et al* in 2016 have published a review of methods of cell immobilization and their applications. Advantages of whole cell immobilization over enzyme immobilization were mentioned in the review. Various techniques of whole cell immobilization with their merits and demerits were also discussed.

Trehan K. in 1997 have mentioned several advantages of immobilized plant and microbial cells such as increased rate of biochemical reactions, increased transport of substances across the membranes, quick and easy recovery of cellular products etc. He has elaborated various methods of immobilization and mentioned entrapment in gels using calcium alginate as a suitable method which has been used in the current study.

Shinke *et al* in 1978 have carried out study on effects of metal ions on determination of β-amylase activity using 3,5 dinitrosalysilic acid(DNSA).Certain metal ions like Mn and Zn were found to enhance the reduction of DNSA by reducing sugar and Ca,Sr,Ba were found to decrease it.

In the present study, effect of whole cell immobilization on the activity of amylase produced by *Bacillus* spp. isolated from garden soil and identified as *Bacillus amyloliquefaciens* was carried out. Compared to enzyme immobilization, whole cell immobilization is comparatively less explored area of research.

2. Materials and Methods

2.1Screening and Isolation of amylase producing *Bacillus* spp.from soil (Padhiar and Kommu, 2016)

Garden soil was diluted upto 10^{-6} in sterile saline. 0.1ml of last three dilutions were spread on sterile nutrient agar. Plates were incubated at 23^{0} C for 24 hours. Isolated colonies were subjected to Gram staining. 20 Colonies showing typical morphology of *Bacillus* spp.i.e. long Gram positive rods in chains

with refractile endospores, were transferred to 0.1ml sterile saline. They were numbered as B1,B2,B3,B4-----B20. From each suspension again 0.1ml of 10^{-6} dilution was spread on sterile starch agar plate (Nutrient agar with 1% potato starch) to get well isolated colonies. Plates were incubated at 23^0 C for 24 hours. After incubation isolated colonies were flooded with dilute iodine solution. The isolate showing largest clear zone of hydrolysis around its colony was selected.

2.2 Identification of the best isolate by VITEK2 automated system.

The isolate showing largest clear zone on starch agar was selected and identified by VITEK2 automated system.For identification total 46 tests were carried out in the system.

2.3Production of amylase by free cells

500 ml sterile nutrient broth with 1% soluble potato starch was inoculated with 5% fresh 24 hours old nutrient broth culture of the best isolate and incubated at 23^0 C on rotary shaker at medium speed for 24 hours. The broth was centrifuged at 3000 rpm for 15 minutes and the supernatant was used as amylase enzyme.

2.4 Whole cell immobilization and production of amylase (Nair *et al*, 2007)

25 ml of 24 hours old culture of the best isolate was mixed with 4% sodium alginate solution in the ratio of 1:2. Then the mixture was extruded into ice cold, 0.2 M $CaCl_2$solution using a sterile syringe to form alginate beads. Beads were allowed to harden in the same amount of fresh ice cold, 0.2 M $CaCl_2$solution for 24 hours in refrigerator. All beads were transferred to 500 ml sterile nutrient broth with 1% starch and kept on rotary shaker at medium speed for 24 hours for production of amylase by immobilized cells. After incubation, beads were removed, broth was centrifuged at 3000 rpm for 15 minutes to remove any free cells and the supernatant was used as amylase enzyme.

2.5 Amylase assay by DNSA method

In the original protocol used by earlier researchers, some modification was done **(**Gusakov *et al*, 2011).

2.5 a. Standard graph Preparation

A standard graph of maltose in µM against optical density (O.D.) at 540 nm was prepared using the following protocol (**Table 1**).

Table 1: Protocol for standard graph preparation for amylase assay

Maltose in µM	Stock solution(10mM=10 µM/ml) of maltose-ml	Distilled water ml
0(Blank)	0	1
1	0.1	0.9
2	0.2	0.8
3	0.3	0.7
4	0.4	0.6
5	0.5	0.5
6	0.6	0.4
7	0.7	0.3
8	0.8	0.2
9	0.9	0.1
10	1	0

After additions, the tubes were kept in boiling water bath for 10 minutes. They were cooled and diluted to 10 ml with distilled water. O.D. was measured using spectrophotometer (Systronic model) at 540 nm against Blank.

DNSA reagent was prepared by mixing 10 gm of 3,5 dinitro salicylic acid dissolved in 200 ml of 2mol/lit solution of NaOH with 300 gm of sodium potassium tartarate dissolved in 500 ml distilled water. The mixture was diluted to 1 litre (Plummer, 2000).

2.5b . Amylase assay was carried out using the following protocol (Table 2)

Table 2: Protocol for Amylase assay

Additions	Blank	Test	Control
1%starch solution	0.5ml	0.5ml	0.5ml
Phosphate buffer pH7	0.4ml	0.4ml	0.4ml
Distilled water	0.1ml	0.1ml	0.1ml
Cell free broth	-	0.1ml	-
	Incubated at 23^0C	for 20 minutes	
DNSA reagent	1ml	1ml	1ml
Cell free broth	-	-	0.1ml

All tubes were kept in boiling water bath for 10 minutes. Cooled, diluted to 10 ml with distilled water. O.D. of 'Test' and 'Control' was measured against 'Blank' at 540 nm.

In this method, the control used was a combined control for substrate and enzyme. Hence, O.D. of 'Control' was subtracted from O.D. of 'Test'. The difference was plotted on the standard graph and extrapolated on X-axis to determine µM of maltose liberated.

The assay was carried out for both enzymes, produced by the free cells and the immobilized cells and enzyme activities were compared. Enzyme activity was calculated as µM of maltose formed by 1ml of enzyme per minute.

2.6 Optimization of parameters for amylase produced by immobilized cells.

Optimization of temperature, pH and substrate concentration was carried out by carrying out assay at pH 5.5,6,6.5,7,7.5, temperature 25^0C, 35^0C, 45^0C, 55^0C, and using 0.25%, 0.5%, 0.75% and 1% starch in nutrient broth used as production medium.

3. Results and Discussion

3.1 Screening and isolation of Amylase producing *Bacillus* spp.

20strains were isolated from garden soil, showing typical morphology of *Bacillus* spp. and producing clear zone on starch agar plate when flooded with dilute iodine solution. The best strain showing largest zone of clearance was B6, which showed 18mm diameter. Hence it was selected and further used. Same method of screening was used by earlier researchers (Padhiar and Kommu, 2016). Majority of researchers have used *Bacillus* spp for production of amylase as the activity of the enzyme produced by members of this genus is found to be significantly high (Pandey *et al*, 2000).

3.2 Identification of the best isolate

When the best isolate was subjected to VITEK-2 automated system of bacterial identification. It was identified as *Bacillus amyloliquefaciens*. Earlier researchers have mentioned several advantages of VITEK-2 automated system for identification of bacteria over conventional methods (Sony and Potty, 2017).

3.3 Assay of amylase using cell free broth obtained using free cells and immobilized cells. All experiments were carried out in triplicate.

3.3.1 Standard graph prepared using maltose, µM Vs. Optical density at 540 nm resulted into a straight line passing through origin (**Figure** 1).

Maltose -µM	Mean O.D. at 540nm
1	0.073
2	0.133
3	0.213
4	0.263
5	0.320
6	0.417
7	0.497
8	0.587
9	0.683
10	0.730

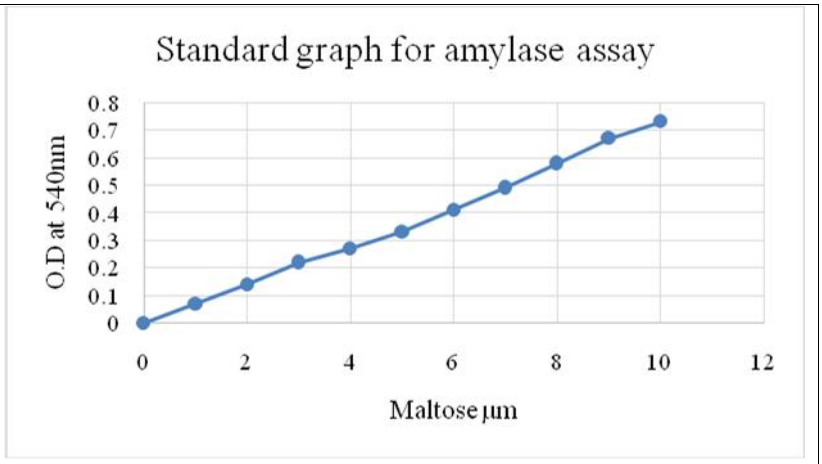

Figure 1

3.3.2Whole cell immobilization of *Bacillus amyloliquifaciens* using calcium alginate Whole cell immobilization of *Bacillus amyloliquifaciens* resulted into firm beads formation(**Figure 2**).When amylase activity of cell free broths obtained by centrifugation of free cells and immobilized cells was compared, amylase produced by free cells showed activity of 2.78 µM/ml/min. Whole cell immobilization of cells using calcium alginate resulted into 2 times improvement of enzyme activity which was 5.53 µM/ ml/ min indicating enhanced production of enzyme (**Figure 3**).

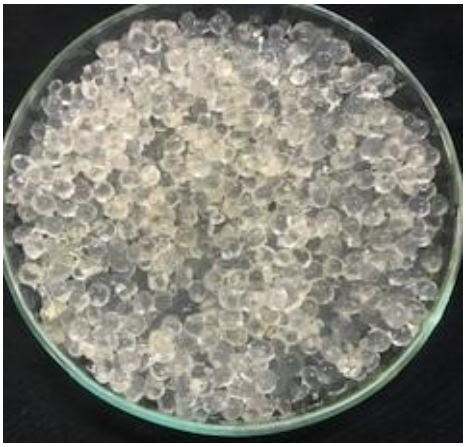

Figure 2

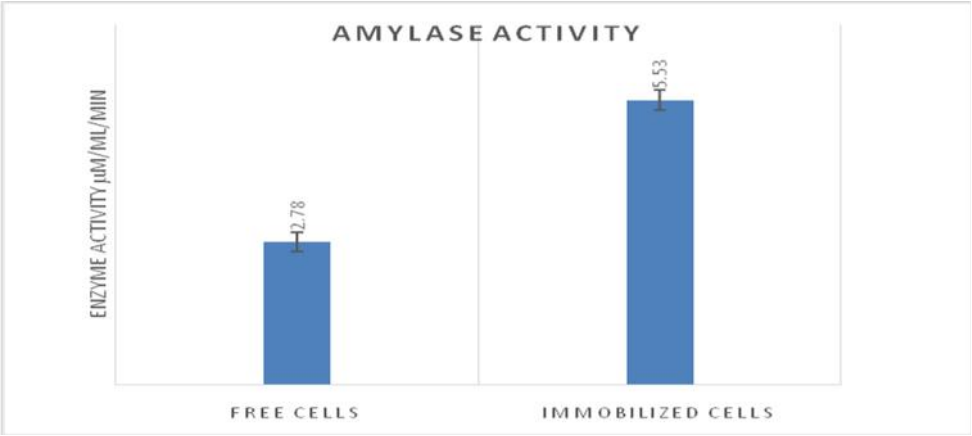

Figure 3

Similar study was carried out by Kokubu *et al* in 1978. They had immobilized *Bacillus subtilis* using polyacrylamide gel and noticed 3 times greater production of α-amylase by immobilized cells than free cells.

Some researchers have noticed elevated production of calcium carbonate by bacterial cells immobilized in the calcium alginate beads fabricated using 1.38% w/v Na-alginate and 0.13 M $CaCl_2$ (Seifan *et al*, 2017).

3.3.3 Optimization of environmental parameters for amylase produced by immobilized cells.

Study of optimization of parameters for enzyme produced by immobilized cells indicated pH 6.5, temperature 25^{0}C and substrate concentration 1% as the most optimum parameters for getting maximum enzyme activity by immobilized cells (**Figures 4a-4c**).

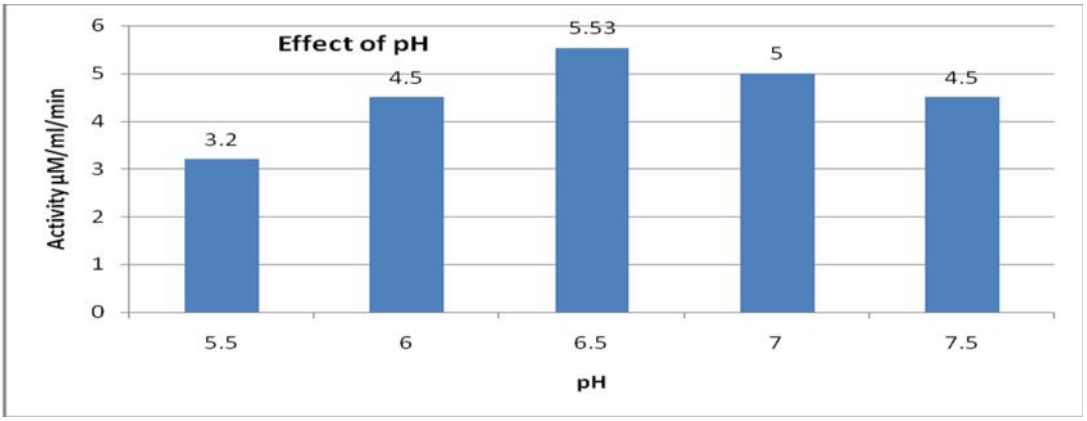

Figure 4a

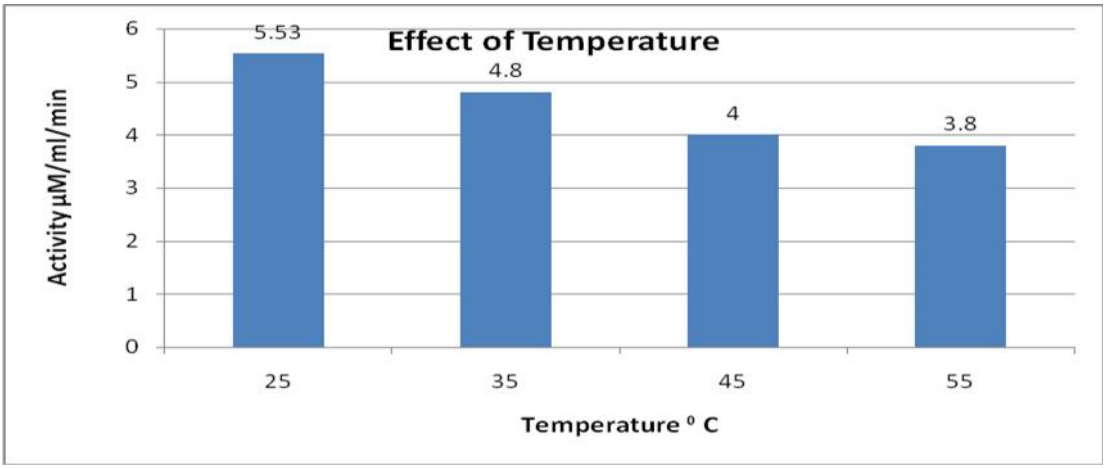

Figure 4b

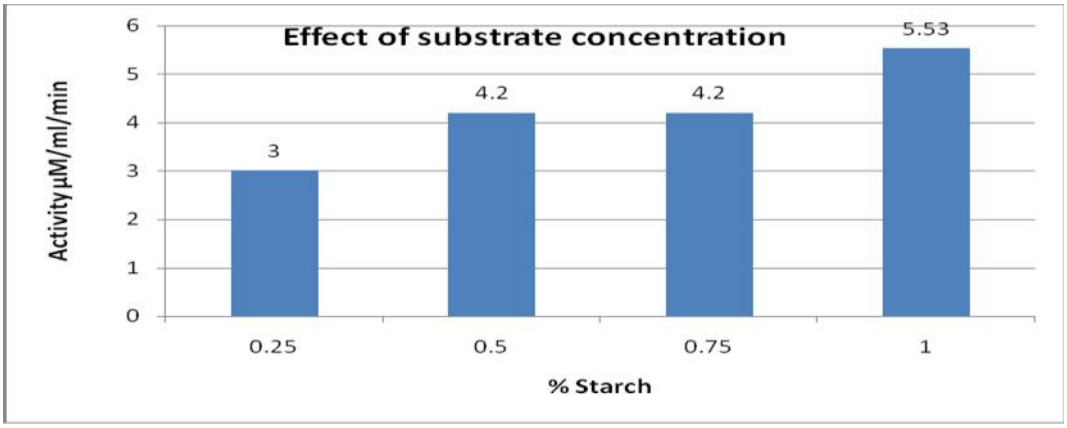

Figure 4c

Salihu *et al* in 2015 have isolated *Bacillus* spp. from soil and identified using 16S-rRNA gene sequencing. Enzyme activity of amylase produced by these isolates was determined by DNS method. Optimization study for enzyme activity was carried out for environmental parameters like temperature, pH etc. The study was similar to the current study but whole cell immobilization was not carried out by the researchers.

Similar optimization study for amylase produced by *Pseudomonas mendocina* was carried out. Researchers have noticed the maximum enzyme production after 72 hoursat temperature 40°C and pH 7. Among various C and N sources,1% glucose and 1 % Tryptone was found to be the best for amylase production. It was found that 0.1% of Mg2+ increased enzyme production whereas other metal ions exhibited inhibitory effects. The enzyme production was maximum at 5% substrate (starch) concentration which shows inducing effect of substrate (Padhiar and Kommu, 2016).

4. Conclusion

Bacillus amyloliquefaciens isolated from garden soil produced extracellular amylase and showed a large clear zone of hydrolysis on starch agar plate. Whole cell immobilization by calcium alginate is a simple technique and has shown two times increase in amylase activity indicating improved production of amylase by the immobilized cells. Hence, the technique may be considered suitable for production of amylase on industrial scale. Knowledge of optimum parameters for the production of amylase by immobilized cells, may be used for application of enzyme on large scale. Further scale up studies are required for large scale production of enzyme by immobilized cells. The enzyme preparation used in the current study was a crude preparation. To improve the enzyme activity, it may be subjected to enzyme purification techniques. Further, the strain may be subjected to strain improvement program for higher production of enzyme.

Statement of conflict of interest

The author declares that there is no conflict of interest.

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