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Potential of Potato Peel as Substrate for Amylase Production in Solid State Fermentation

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Abstract

Microbial amylases have extensive commercial applications in various sectors such as food, beverages and pharmaceutical industries. In the present study, amylase producing bacterial strain was isolated from soil sample collected from garden campus of S. M. Joshi College, Hadapsar, Pune, Maharashtra. Primary screening of amylase producers was done on agar plates containing starch as substrate. Among 6 isolates, the potential strain was identified as *Bacillus subtilis* based on morphological cultural and biochemical tests. The solid-state fermentation was carried out using 20 % potato peel as substrate. The optimized physicochemical parameters were identified as pH 7, room temperature (~30 °C), 48 h and 7.5 % substrate concentration. The amylase activity was further improved in presence of 0.2 % (w/w) peptone, 0.02 % $MgSO_4 \cdot 7H_2O$, 0.04 % $CaCl_2 \cdot 2H_2O$ and 0.04 % KH_2PO_4. Additionally, the optimized physicochemical and nutritional parameters reduced the fermentation time period by 50 % and gave optimum yield of enzyme in 24 h. Thus, the present study highlights the potential of *B. subtilis* strain to produce significant yield of amylase using agricultural wastes like potato peels.

Keywords: Potato peel; *Bacillus subtilis*; Amylase; Solid state fermentation; Optimization; Agricultural wastes.

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1. Introduction

Amylases (E.C.3.2.1.1) are among the first commercially produced enzymes. They capture approximately 25 % of the global enzyme market, signifying their competent market value [1]. For decades, they have been used in pharmaceutical, detergent and food industries. Presently, they are gaining more importance due to their biotechnological potential in pretreatment of activated sludge to enhance biodegradability [2]. Additionally, they are also used for pretreatment of animal feed to improve digestibility. Amylases act as a biocatalyst for the conversion of starch into valuable products by hydrolysis of internal α-1,4-glycosidic

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linkages. Hence, they are commonly applied in industrial processes like liquefaction of starch, as a digestive aid and clarification of beer or fruit juices [3,4].

Sources of amylase include bacteria, fungi, plants and animals. Among these, bacterial amylases have gained more attention in the recent years due to its ease of production, stability of enzyme at high temperatures, simple and cost-effective processing and potential of genetic engineering to obtain enzymes of desired characteristics [5,6]. In contrast, fungal species suffer drawbacks like slow growth of culture, lower yield and difficulty in purification of enzymes from growth medium [7]. Microbial sources of amylase are currently acknowledged for improving fermentation processes, extending shelf lives and increasing softness of the baked product in food industries. Besides, they are also applied in the production of sugar syrups [4,8]. Considering the diversity of bacterial species and their metabolic products, screening of potential environmental isolates are persuasively encouraged for economical outcomes. Currently, bacterial amylases are effectively produced by solid-state fermentation [9-11].

Despite the many advantages, bacterial fermentation processes pose challenges in sufficient scale up to meet the industrial demands. One major drawback of large scale microbial fermentation set up is the need for large quantity of substrates. Although purified carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are feasible for laboratory processes, they are very expensive for commercial production of enzymes. This drawback can be overcome with the use of agricultural wastes such as millet starch, potato and wheat bran. Precisely, potato (*Solanum tuberosum*) skin is an attractive alternative substrate due to the presence of considerable amount of starch [12,13]. On a dry weight basis, potato wastes contain approximately 3.4 % pectin, 2.2 % cellulose, 14.7 % protein, 66.8 % starch, and 7.7 % ash [14]. Also, potatoes are the world's fourth-largest food crop, following maize (corn), wheat and rice. The agricultural wastes of other 3 sources contain negligible amount of starch [15].

To set up a microbial fermentation process, valuable insights can be gained from published literature. For instance, it is well established that optimizing the growth conditions and nutrient concentrations can significantly promote the yield of microbial amylases to several folds [9-12]. Also, agricultural wastes, that otherwise accumulate in the environment causing pollution, can be aptly diverted towards fermentation processes in the form of substrates [9-11,16-18]. Although, considerable attempts are made to utilize agricultural wastes as animal feed, there is a limit to which the massive quantities of generated wastes can be repurposed. Hence, given the potential of bacteria to metabolize nutrients at extremely high rate, the biotechnologically optimized fermentation processes can prove to be an excellent means of sustainable industrial approach towards green technology. Useful insights into using potato peel as valuable substrate for amylase production came from early studies that indicated their suitability in fermentation [19-21]. Other than *Bacillus* sp., *Lactobacillus, Escherichia, Proteus, Streptomyces* and *Pseudomonas* have been reported to produce amylases [22-24]. Literary evidence also highlights the abundance of amylase producing *Bacillus* sp. [25-27]. This may be due to the fact that *Bacillus* is a common environmental flora prevalent in soil, and can survive on dust

particles. Their rapid growth rate and capacity to secrete extracellular proteins offer additional benefits in fermentation processes due to short fermentation cycles and improved enzyme recovery respectively [26].

Considering these factors, we intended to incorporate the above beneficial characteristics in fermentation process to improve the yield of α-amylase. Hence, the objective of the present investigation was to employ potato peel waste as solid substrate for production of α-amylase from *Bacillus* sp., isolated from soil, and optimize the fermentation process.

2. Materials and Methods

2.1. *Sample collection*

The soil sample used in our study was obtained from the garden campus of S.M. Joshi College, Hadapsar, Pune, Maharashtra. The sample was collected in sterile containers with the help of pre-sterilized spatula and stored in UV sterilized plastic bag in cool and dry place until further use.

2.2. *Screening and isolation of amylase producing bacteria*

The soil sample (1 g) was suspended in 10 mL of sterile phosphate buffer (pH 7.2) and serially diluted to obtain countable colonies on Nutrient Agar (NA) medium by spread plate method. The plates were incubated at 37 °C for 24 h. After incubation, well isolated colonies were spot inoculated on agar plates containing 1 % starch and further incubated at 37 °C for 24 h. The screening of amylase producing isolate was done by flooding the above plates with Lugol's iodine solution (w/v 1 % iodine in 2 % potassium iodide). The hydrolysis of starch around the colony forming a zone indicated amylase production [28]. The colonies with wider halo zone were suggestive of higher enzyme activity. Hence, they were re-isolated and maintained as pure cultures for further studies.

2.3. *Morphological and biochemical characterization*

The isolated strains were identified based on morphological, cultural and biochemical tests as indicated in Bergey's Manual [29].

2.4. *Enzyme assay*

The cells were grown in growth medium and mixed with 100 mL of sodium phosphate buffer (pH 6.9). The flasks were incubated on shaker at 150 rpm for ½ h and the medium was filtered through Whatman Filter Paper No.1. The filtrate was chilled in refrigerator and centrifuged at 1000 rpm for 10 min. The supernatant was collected carefully and used as

crude enzyme extract. Sugar estimation and determination of enzyme activity was done using the DNSA [30] method and proteins were estimated by Folin Lowry [31] method.

2.5. *Optimization of amylase production by potential bacterial isolate*

2.5.1. *Preparation of substrate*

The potatoes were purchased from local market and its peels were used as substrate in this study. The peels were spread on trays and dried in oven at 70 °C for 24 h. The dried peels were ground in mixer grinder (Videocon) and stored in polythene bags at room temperature (25 ± 1 °C).

2.5.2. *Preparation of inoculum*

The isolate was grown in fermentation medium [composition (in g/L): glucose (20), yeast extract (3), peptone (5), NaCl (15), disodium hydrogen phosphate (15), di-hydrogen sodium phosphate (3), potassium chloride (0.1), magnesium sulphate (0.1)] at 37 °C for 24 h under shaker conditions. A homogenous suspension (5 mL) of 0.1 $O.D_{540nm}$ (approximately 10^6-10^7 cells/mL) was used as inoculum.

2.5.3. *Solid state fermentation*

For solid state fermentation process, 200 mL fermentation medium containing 20 % starch was used. A control set up was also maintained using 100 mL distilled water with 20 % starch. The flasks were incubated under static conditions.

Most bacterial enzymes are extracellular in nature and their production as well as activity is greatly influenced by nutritional and physico-chemical factors. For optimization process, the fermentation was carried out in 1 % starch broth by applying one factor at a time (OFAT) approach. Using this approach, the variables of one parameter is studied by keeping the others constant. The varying physico-chemical parameters optimized in our study included the incubation period (24 h, 48 h, 72 h), temperature (30 °C, 37 °C), pH (5-9) and substrate concentration (5 %, 7.5 %, 10 %). In addition, varying concentrations of peptone (0.1 % - 0.5 %), magnesium sulphate (0.01 % - 0.05 %), calcium chloride (0.01 % - 0.05 %) and dihydrogen potassium phosphate (0.01 % - 0.05 %) were analyzed to observe its effect on enzyme production.

2.6. *Statistical analysis*

All the experiments were carried out in triplicate, and the results were reported as mean values with standard deviation.

3. Results and Discussion

3.1. *Screening and identification of amylase producing bacteria*

On screening, 6 colonies showed presence of halo zones on starch agar plates, indicating the production of amylase. Among these isolates, the colony showing a large and clear halo zone was selected for further studies. It was identified as *Bacillus subtilis* based on morphological, cultural and biochemical tests. Under un-optimized conditions, the maximum enzyme activity was observed on 48 h incubation.

The bacterial strains capable of producing valuable organic acids, enzymes and proteins are the backbone of biotechnology. The genus *Bacillus* is known for its ability to produce a diverse variety of extracellular enzymes [32,33]. Hence, practical outcomes are more commonly attained by employing *Bacillus* sp. in fermentation processes.

3.2. *Optimization of physicochemical parameters to enhance amylase production*

The hydrolytic enzymes often produce organic acids as a byproduct which accumulates in the fermentation medium lowering its pH. A higher concentration of substrate leads to this transformation at a faster rate. This causes cell death, or reduction in the density, of inoculum [34]. Conversely, lower concentration of substrate and low initial inoculum density slows down the fermentation process [11]. Besides pH and substrate concentration, temperature also plays a key role during fermentation due to its influence on microbial metabolism and maintenance of osmosis [35]. Hence, it is important to optimize these parameters for maximizing the potential of bacterial strains to produce primary or secondary metabolites including amylase. Fig. 1 represents the optimum physicochemical parameters for amylase production by *B. subtilis* strain isolated in this study. These parameters were identified as pH 7, room temperature (~30 °C) and 7.5 % substrate concentration. As a general trend of fermentation phases, the yield and enzyme activity were low during the initial lag phase of growth. It peaked at 48 h incubation after which there was a significant decline. As discussed earlier, this observation may be due to various factors including exhausted nutrients or accumulated byproducts [11,34].

Similar to our study, the OFAT optimization method was adopted by Rehman *et al*. [36] to maximize amylase production by *Bacillus cereus* AS2 strain. They reported pH 7.0, 45 °C and 72 h incubation time as optimum parameters in their study that yielded ~ 1000 U/mL/min. Comparatively, lower incubation time was reported by Abdel-Fattah *et al*. [37] for optimum amylase production from *Bacillus licheniformis* AI20 which showed an enzyme activity of 384 U/mL/min. A *B. subtilis* strain isolated from hot water springs in Gazan city, Saudi Arabia showed expected characteristics of growth and enzyme production. The optimum parameters were reported as pH 8.5, 45 °C, 48 h and shaker conditions that resulted in enzyme activity of 25 U/mL [28].

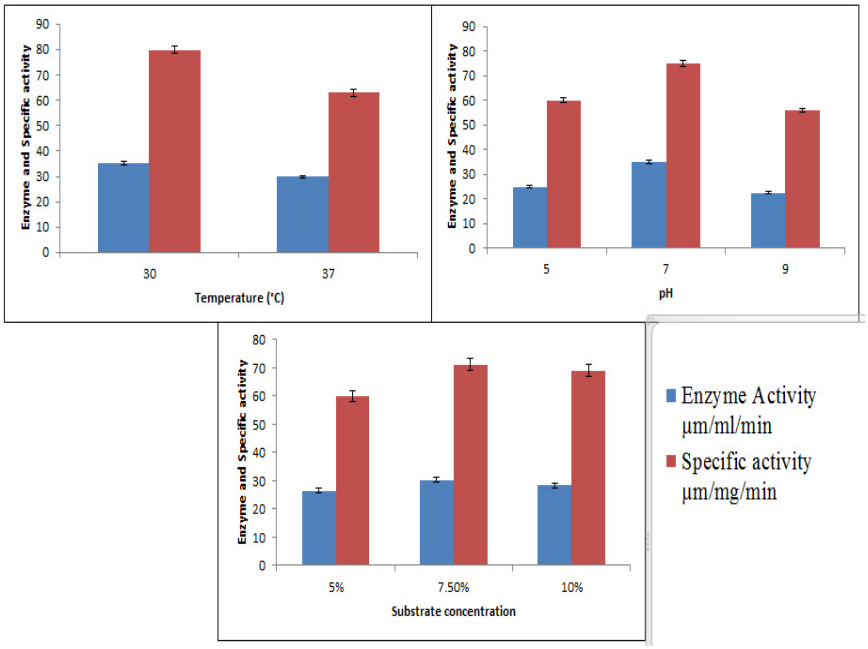

Fig. 1. Optimization of physicochemical parameters for amylase production.

3.3. Optimization of nutritional parameters to enhance amylase production

In addition to the physicochemical parameters, nutritional factors influence microbial growth. In turn, it affects physiological pathways that regulate enzyme production and activity. Precisely, the nitrogen and carbon sources are fundamental nutrients for sustained metabolism [38]. Since every environmental strain is adapted to different conditions, they respond uniquely to various nutrients. For the same reason, a standard protocol cannot be generated for microbial processes and studies are required to understand the optimum nutritional and physicochemical conditions for production of secondary metabolites by every environmental strain. In the present study, the most common carbon and nitrogen sources known to promote amylase production were selected and their concentrations were optimized. Fig. 2 represents the optimum nutritional parameters for maximizing amylase production and activity by *B. subtilis* strain isolated in this study. The results showed optimum amylase activity in presence of 0.2 % (w/w) peptone, 0.02 % $MgSO_4.7H_2O$, 0.04 % $CaCl_2.2H_2O$ and 0.04 % KH_2PO_4.

Supplementation of fermentation medium with inorganic nitrogen (0.05 % $CaCl_2$) sources has also been reported to promote amylase production in *B. cereus* AS2. In addition, it was also stimulated on addition of 0.5 % glycine in the same study [36]. In another study, peptone was reported to be a better inducer of amylase as compared to inorganic nitrogen sources [28]. Similar observations were also reported by Fooladji and Sajjadian [39].

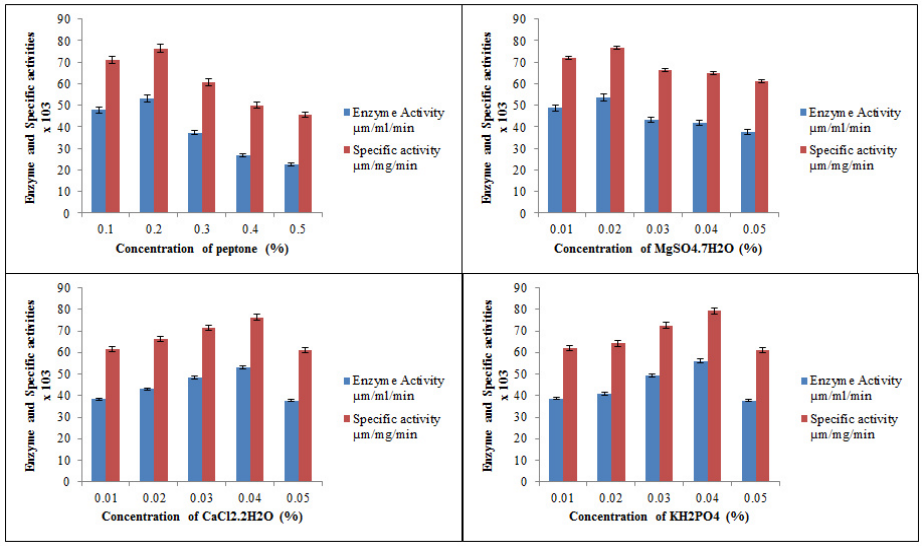

Fig. 2. Optimization of nutritional parameters for amylase production.

3.4. *Solid state fermentation for amylase production under optimized conditions*

Under optimized conditions, 50 % reduction in incubation time was observed i.e., maximum yield and enzyme activity was observed in 24 h (Fig. 3); whereas, under un-optimized conditions, optimum enzyme activity was observed in 48 h (Fig. 1). The enzyme activity observed under optimized conditions was 7.93×10^6 IU/mL/min.

As a modified approach, a recent study used a two-stage statistical method for optimization of amylase production from halophilic *Bacillus* sp. H7. They reported a 1.31-fold increase in amylase production using 5 L laboratory-scale bioreactor for solid state fermentation. The catalytic efficiency of the enzyme was also reported to be significantly high (13.73 mL/s/mg) [40]. Similarly, improvement in fermentation was also achieved by using Response surface methodology in another study [41]. Among other substrates, a study reported use of banana fruit stalk for amylase production. Although the enzyme yield was low (5.3×10^6) compared to our study, it is a feasible approach for sustainable enzyme production. The optimum parameters reported in their study was pH 7.0, 35 °C, 10 % substrate and 24 h. Nutrients like ammonium sulphate/sodium nitrate (1 %), beef extract/peptone (5 %), glucose/sucrose/starch/maltose (0.1 %) and potassium chloride/sodium chloride (1 %) further supported the enzyme production. Moreover, the study reported 2.65-fold higher amylase yield on use of banana fruit stalk as compared to wheat bran [42].

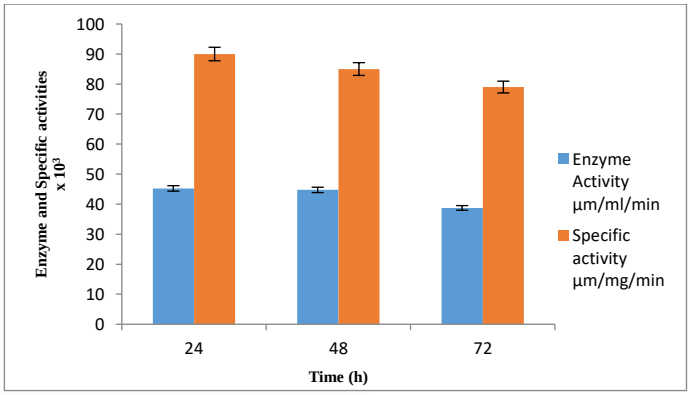

Fig. 3. Amylase activity under optimized conditions.

4. Conclusion

The high costs associated with enzyme production demands alternative strategies for meeting the high requirement of valuable enzymes like amylase for commercial use. The use of potato peels as a substrate in bacterial fermentation is a cost effective and sustainable approach with potential scope to overtake the harmful chemical enzyme production techniques. In the present study, simple techniques were employed to optimize the fermentation process which resulted in significant increase in amylase yield from *B. subtilis* strain accompanied by significant decrease in incubation time. This suggests the immense potential of the isolated strain in biotechnology. Moreover, we employed potato waste as substrate to induce enzyme production. Potato peel is rich in starch and other organic nutrients and hence serves as an excellent carbon source that can induce robust microbial growth and enhance enzyme production. This further adds to the significance of this study that combines two individual sustainable aspects of utilizing cost effective natural raw materials and waste management into a single approach. Such applications not only benefit the industry but also the environment. Overall, the enhanced amylase yield observed in our study can be attributed to several inter-connected factors such as the availability of easily metabolizable starch from potato peel, the optimized physicochemical and nutritional conditions, and the adaptability of *B. subtilis* to grow in medium containing complex organic substrates. It may be possible that the starch from potato peel likely stimulates the microbial enzymatic pathways in some ways, leading to increased amylase secretion. However, more studies are needed to confirm this hypothesis. Additionally, the balanced supply of nitrogen and trace elements may have created an environment conducive to high metabolic activity and enzyme synthesis.

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