International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 05-01-2022, Accepted: 20-01-2022, Published: 05-02-2022
Volume 7, Issue 1, 2022, Page No. 185-189

Studies on the optimization of cultural conditions for the production of amylase by *Bacillus* sp

N Pandeeswari, N Kiruthiga
Department of Agricultural Microbiology, Faculty of Agriculture, University, Annamalai Nagar, Chidambaram, Tamil Nadu, India

Abstract
Amylases are one of the most widely used enzymes in agriculture. These enzymes are crucial in hydrolyzing starch molecules into polymers made up of glucose units. Amylases could be used in a wide range of commercial operations, including the food, fermentation, and pharmaceutical industries. Despite the fact that amylases can come from a variety of places, including plants, animals, and bacteria, microbial enzymes typically suit industrial needs. Cassava waste was used to isolate the bacteria. *Bacillus amyloliquefaciens, Bacillus licheniformis, and Bacillus subtilis* were identified using staining techniques, motility tests, plating on selective media, and biochemical assays. After incubation, the blue colour of the starch agar media around the microbial colonies disappeared, indicating that these *Bacillus* spp. were producing amylase. The substrate for the synthesis of amylase was cassava waste. Using these *Bacillus* spp., solid state fermentation was used to produce amylase. The influence of temperature and pH on enzyme synthesis by *Bacillus* spp. was investigated in this work. Amylase activity is measured using techniques such as starch-iodine colour intensity reduction and plate test.

Keywords: cultural conditions, production, *Bacillus sp*

Introduction
Amylolytic enzymes that degrade starch are important in biotechnological applications ranging from food to fermentation, textiles to paper (Lin *et al*., 1997; Pandey *et al*., 2000) [15]. Amylases come from a variety of places, including plants, animals, and microorganisms. Microbial amylases satisfy industrial needs; an excessive number of them are commercially available; and they have nearly totally supplanted chemical starch hydrolysis in the starch processing sector (Pandey *et al*., 2000) [15]. The main benefit of employing microorganisms to make amylases is the low cost of mass manufacturing and the ease with which microbes can be controlled to produce enzymes with desired properties (Lonsane and Ramesh, 1990). Amylase has been developed from a variety of bacteria, fungi, yeasts, and actinomycetes, however enzymes obtained from bacteria and fungi have dominated uses in the industries (Pandey *et al*., 2000; Ahn *et al*., 2015) [1, 15].
Solid state fermentation (SSF) has gotten a lot of attention in recent years as a low-cost alternative for enzyme manufacture and application (Govarthanan *et al*., 2014; Govarthanan *et al*., 2015) [9, 18, 10]. Agro-industrial leftovers are commonly regarded as suitable substrates for the production of value-added goods (Selvankumar *et al*., 2011). Cassava (*Manihot esculenta*) is a starch-rich crop farmed primarily in tropical regions such as Asia. It is used for human food, animal feed, and starch extraction. Protein, lipid, lignocellulosic fibres, and sugars were also present (Teixeira *et al*., 2009) [21]. To maximise the effective exploitation of cassava waste, it can be biotechnologically turned into a variety of value-added products (Pandey *et al*., 2000) [15]. The bioconversion of cassava waste into protein, biomolecules, organic acids, and food components, among other things, has been examined as an industrial development for the use of cassava waste (Soccol and Vandenberghe, 2003) [20]. As a result, the main purpose of the research is to isolate and identify amylolytic bacteria from cassava waste, as well as to characterise the enzyme production and characteristics in relation to temperature and pH.

Materials and Methods
Substrates used
Cassava waste (Solid waste) was obtained from Sago processing industry, Salem, Tamil Nadu, India.

Isolation of *Bacillus* spp.
Amylolytic bacteria will be isolated from Cassava solid waste (Cassava baggase). Bacterial isolation will be done on nutrient agar plates using the serial dilution method. In this situation, bacteria will be inoculated on NA medium using 0.1 ml of dilution from 10^{-5} of the sample. The inoculated media will be incubated for 24 hours at 37 degrees Celsius. Discrete colonies will be counted and recorded as colony forming units per millilitre (cfu ml^{-1}). To obtain the pure culture, the isolates will be sub cultured on slants. The slants will be kept at 4°C.

Screening and Identification of amylase producers

Isolated bacterial colonies maintained at 4°C, will be further evaluated for amylolytic potential by inoculating them in a starch agar plate. The inoculated plates will be incubated for 3 days at 37°C. By flooding the plates with Gram's iodine solution (1 g of iodine crystals and 2.0 g of potassium iodide will be dissolved in 100 ml of distilled water, stored at room temperature) after 3 days of incubation, amylolytic bacteria will be detected (Pokhrel and colleagues, 2013).

Characterization of bacterial isolates

Starch and iodine interacted to form a dark blue starch-iodine combination that filled the entire agar plate. When inundated with Gram's iodine solution, the positive colonies showed a definite zone of hydrolysis around the colonies. Against a blue-black colouring on starch agar, the negative colonies generated no hydrolysis zone surrounding them. (Benkiar and colleagues, 2013; Kaur and colleagues, 2012; Parmar and colleagues, 2012) [3, 16].

Optimization of culture conditions

The effect of culture conditions in this study were administered at various temperatures (30, 35, 40, 45, 50, 55, and 60°C) and pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) with the goal of producing a large amount of amylase enzyme. (Irfan and colleagues, 2011).

Solid state fermentation

A bacterial amylase production using (g/l) KH_2PO_4 – 1.4; NH_4NO_3 – 10; KCl – 0.5; $MgSO_4.7H_2O$ – 0.1; $FeSO_4.7H_2O$ – 0.01; starch – 20 gm; and distilled water will be added to the substrate (Cassava Solid Waste) in a 250 ml (flask) Erlenmeyer flask The contents of the flasks will be well mixed before being autoclaved for 20 minutes at 121°C. Solid state fermentation will be carried out at 30°C for 72 hours with a substrate moisture content of 64% and 2 ml of each *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Bacillus subtilis* suspension as inoculum. (Castro and colleagues, 2010) [6].

Enzyme extraction

The cultures were diluted with 22 ml of 0.1 M phosphate buffer (pH-6.5) and then shaken for half an hour at 20°C and 140 rpm on a rotary shaker. After that, the mixture was filtered through cheese cloth and centrifuged for 15 minutes at 8000 rpm at 4°C. The supernatant was collected after being filtered with Whattman Number-1 filter paper. For further research, the filtrate was employed as a crude enzyme preparation.

Estimation of amylase activity

α - amylase activity was assessed by determining the rate at which maltose is released from starch, which is assessed by its ability to degrade 3,5 dinitrosalicylic acid (DNSA). At 25°C, one unit of - amylase activity is the quantity of enzyme that releases 1mg of maltose per minute. In phosphate buffer, the substrate, 1 percent cassava baggase, was gelatinized. The reaction mixture contained 1 mL enzyme solution and 1 mL substrate, which was incubated for 5 minutes at 25°C before being stopped with 1 mL DNSA colour reagent. The mixture was heated for 5 minutes in a water bath at 100°C, then cooled and diluted with 10ml distilled water. The reaction mixture was allowed to stand for 15 min at room temperature and the optical density read at 540nm. A unit of amylase activity was expressed as:

$$\text{Enzyme activity (Units/ml)} = \frac{\mu g \text{ of maltose released}}{\text{Volume of enzyme taken (1 ml)} \times \text{Time of incubation}}$$

Assay of enzyme activity

The DNSA method was used to calculate amylase activity (3,5 dinitrosalicylic acid method). Three test tubes were used (one control and two tests T1 and T2). 1.0 mL phosphate buffer (pH 6.8), 0.5 mL starch, and 1.5 mL enzyme solution were added to the test tubes. No enzyme solution was used in the control group, and 1.5 mL of phosphate buffer was added.

For 30 minutes, the reaction mixture was incubated at 50°C. The reaction was stopped using 2ml of DNS and a 10-minute immersion in a boiling water bath. A spectrophotometer was used to measure the absorbance at 540nm, with glucose as the standard.

Result and Discussion

Other successful studies that intended to create α - amylase utilising agricultural-based media for fermentation, such as Cassava waste, produced similar findings (Zusfahair *et al*., 2016). *Bacillus amyloliquefaciens, Bacillus licheniformis,* and *Bacillus subtilis* (Kumar *et al*., 2012) [14] synthesised Amylase enzyme from cassava waste from the Sago processing industry in Salem. Garg and colleagues (2010) Singh *et al*., 2016 used iodine on starch agar media to validate the assembly of amylase enzyme from *Bacillus* isolates, as well as the development of a clear zone around bacterial growth, which suggests amylase activity. These amylases are examples of hydrolases and their performance during molecular hydrolysis. Amylases are one of the most important enzymes found in

biotechnology. In the food industry, they are used in the hydrolysis of starch to produce glucose syrup, amylase-rich flour, and the creation of dextrin during baking. Amylases are also utilised in the textile industry to remove starch sizing and as detergent additives, among other applications.

The bacterial strain that produced more amylase in this investigation had the following preliminary characterization: gram-positive spore-forming bacilli, around one micron length, with core spores that were usually smaller than the cell. Agar colonies could be small, smooth, shiny, and spherical, or larger, flat, rough, and uneven, or even huge, spreading, and mucoid. The majority of isolates ferment glucose and sucrose while generating acid. Lactose wasn't fermented. Within 72 hours, the majority of them had totally dissolved gelatin. Originally, they all appear to belong to the *Bacillus* sp. group. Gram staining, motility test, selective medium, and biochemical tests were used to isolate and characterise *Bacillus* spp. (*B.amyloliquefaciens, B.licheniformis* and *B.subtilis*).

Amylase activity had first been determined by plating each *Bacillus* spp. on Starch agar medium and flooding the medium with iodine indicator. A clear zone around the colony showed the existence of amylase activity. The bacteria genus is diverse, and its members are adaptable to their surroundings in a variety of ways. The type of their metabolic processes and enzymes generated is influenced by a number of factors. Thermophilic and highly thermophilic bacteria, as well as their enzymes, are receiving a lot of attention. *Bacillus* species produce a larger version of extracellular enzymes like as amylases, which are important in industry. In addition, bacterial enzymes are known to be more thermostable than fungal amylases.

The effect of temperature on amylase production is related to the organism's growth. α - amylase has been produced by bacteria at a far wider range of temperatures. *Bacillus amyloliquefaciens* has been shown to produce amylase continuously at 36°C. Bhange *et al*., 2016 [4] found that the optimal temperature for *Bacillus subtilis* was 70°C using Bacillus cereus, Vijayaraghavan *et al*. (2015) [22] discovered that the optimal temperature is 50°C. Awasthi *et al*., 2018, discovered that the optimal temperature for amylase production in *Bacillus cereus* and *Bacillus licheniformis* was 60°C. Some of the conditions discovered in prior works were similar to those seen throughout this project. There are still some differences, which is due to the fact that each organism, even within the same Genus, has various genes that encode amylase synthesis, hence the behaviour of each amylase under different conditions will be varied as well.

Table 1: Effect of temperature on amylase production

Temperature (°C)	Enzyme activity (U/ml)		
	B.amyloliquefaciens	***B.licheniformis***	***B.subtilis***
30	423	377	655
35	510	415	725
40	734	749	854
45	895	875	737
50	771	926	578
55	395	676	413
60	386	598	392

The effect of temperature on amylase production by *Bacillus amyloliquefaciens, Bacillus licheniformis* and *Bacillus subtilis* utilising cassava waste as a substrate was investigated at 30, 35, 40, 45, 50, 55, and 60°C. In this study, *B. licheniformis* recorded highest enzyme production at 50°C (926 Uml^{-1}) and the lowest enzyme production at 30°C (377 Uml^{-1}) (Table 1).

The pH of the expansion medium is one of the physical characteristics that has a significant impact on the organism's morphology and enzyme secretion. The pH variation noticed throughout the organism's expansion has an impact on product stability in the medium. The majority of *Bacillus* strains utilised commercially for the assembly of bacterial α - amylases by SmF have an optimal pH for growth and enzyme synthesis between 6.0 and 7.0. This is generally true of the strains used by SSF to assemble the enzyme. Except for pH 6.8 for *Bacillus amyloliquefaciens*, the pH utilised is rarely given. Using *Bacillus subtilis*, Bhange *et al*., 2016 [4] obtained an optimal pH of 6.0. Using *Bacillus cereus*, Vijayaraghavan *et al*. (2015) [22] discovered that the optimal pH is 7.0. Awasthi *et al*., who studied amylase production in *Bacillus cereus* and *Bacillus licheniformis*, discovered that *B. cereus* amylase has a pH of 6.5 while *B. licheniformis* amylase has a pH range of six to eight.

Table 2: Effect of pH on amylase production

pH	Enzyme activity (U/ml)		
	B.amyloliquefaciens	***B.licheniformis***	***B.subtilis***
3.0	386	418	455
4.0	433	507	575
5.0	682	689	630
6.0	723	910	740
7.0	842	725	782
8.0	580	417	351

9.0	477	397	322

In the present study, the effect of pH on amylase production by *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus subtilis* was carried out at various pH *viz*., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The enzyme production was maximum at pH 6.0 (910 Uml^{-1}) recorded by *B. licheniformis* and minimum at pH 9.0 (322 Uml^{-1}) recorded by *B. subtilis* (Table 2).

Conclusion

Over the last five decades, amylase research has advanced at a breakneck pace, with potential industrial uses, particularly in solid waste management, emerging. The yield, stability, and cost of amylase manufacturing are all major barriers to realising the commercial potential of the enzyme. Although many researchers have investigated microorganisms extensively, the natural bacterial flora of the cassava baggase, gathered from the Salem agro-based industry, was discovered for amylase production in this study. A total of 15 bacterial isolates were tested for extracellular amylase production in starch agar medium, and 5 of them were reported to be positive based on the formation of a hydrolysis zone in starch agar plates. *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus subtilis* were employed to produce amylase, with cassava waste as a substrate in solid state fermentation. The selected bacterial isolate with high amylase activity can be further characterised for various industrial applications.

References

1. Anh DBQ, Mi NTT, Huy DNA, Hung PV. Isolation and optimization of growth condition of *Bac*illus sp. from fermented shrimp paste for high fibrinolytic enzyme production. Arab. J. Sci. Eng,2015:40:23-8.
2. Awasthi MK, Wong JW, Kumar S, Awasthi SK, Wang Q, Wang M *et al*. Biodegradation of food waste using microbial cultures producing thermostable α-amylase and cellulase under different pH and temperature. Bioresource technology,2018:248:160-170.
3. Benkiar A, Nadia ZJ, Badis A, Rebzani F, Soraya BT, Rekik H *et al*. Biochemical and molecular characterization of a thermo and detergent-stable alkaline serine keratinolytic protease from *Bacillus circulans* strain DZ100 for detergent formulations and feather- biodegradation process. Int. Biodeterior. Biodegrad,2013:83:129-138.
4. Bhange K, Chaturvedi V, Bhatt R. Simultaneous production of detergent stable keratinolytic protease, amylase and biosurfactant by *Bacillus subtilis* PF1 using agro industrial waste. *Biotechnol Rep*,2016:10:94:104.
5. Bozic N, Ruiz J, Lopez-Santin J, Vujei Z. Production and properties of the highly efficient raw starch digesting -amylase from a Bacillus licheniformis ATCC 9945a. Biochem. Eng. J,2011:53:203-209.
6. Castro AM, Carvalho DF, Freire DMG, Castilho LR. Economic analysis of the production of amylases and other hydrolases by Aspergillus awamori in solid state fermentation of babassucake. Enzyme Res,2010:1:9.
7. Dogan IS. Effect of α amylases on dough properties during Turkish hearth bread production. International journal of science & technology,2003:38(2):209-216.
8. Garg D, Kaur M. Extraction, purification and characterization of enzyme amylase from Bacillus amyloliquefaciens. Int. J. Adv.Eng.Sci,2013:3(3):158-159.
9. Govarthanan M, Park SH, Kim JW, Lee KJ, Cho M, Kamala-Kannan S *et al*. Statistical optimization of alkaline protease production from Brackish Environment Bacillus sp. SKK11 by SSF using horse gram husk. Prep. Biochem. Biotechnol,2014:44:119-131.
10. Govarthanan M, Selvankumar T, Selvam K, Sudhakar C, Vincent Aroulmoji, Kamala-Kannan S. Response surface methodology based optimization of keratinase production from alkali-treated feather waste and horn waste using Bacillus sp. MG-MASC-BT. J. Ind. Eng. Chem,2015:27:25-30.
11. Irfan M, Gulsher M, Abbas S, Syed Q, Nadeem M, Baig S. Effect of various pretreatment conditions on enzymatic saccharification. Songklanakarin J. Sci. Technol,2011:33(4):397-404.
12. Karnwal A, Nigam V. Production of amylase by isolated microorganisms and its application. Int. J. Pharm Bio. Sci,2013:3(4):354-360.
13. Kaur A, Kaur M, Samyal ML, Ahmed Z. Isolation, characterization and identification of bacterial strain producing amylase. J. Microbiol. Biotech. Res,2012:2(4):573-579.
14. Kumar MDJ, Priyadharshini AD, Suresh K, Saranya GM, Rajendran K, Kalaichelvan PT. Production, Purification and Characterization of α-Amylase and Alkaline Protease by Bacillus sp. HPE 10 in a Concomitant Production Medium. Asian J. Plant Sci. Res,2012:2(3):376-382.
15. Pandey A, Soccol CR, Nigam P, Soccol VT, Vandenberghe LPS, Mohan R. Biotechnological potential of agro industrial waste, cassava baggage. Biotechnology source,2000:74(1):81-87.
16. Parmar D, Pandya A. Characterization of Amylase Producing Bacterial Isolates. Bull. Environ. Pharmacol. Life Sci,2012:1(6):42-47.
17. Pokhrel B, Wanjare P, Singh S. Isolation, screening and characterization of promising-amylase producing bacteria from sewage enriched soil. Int. J. Adv. Biotechnol. Res,2013:4(2):286-290.
18. Selvam K, Govarthanan M, Kamala-Kannan S, Govindharaju M, Senthilkumar B, Selvankumar T *et al*. Process optimization of cellulase production from alkali-treated coffee pulp and pineapple waste using Acinetobacter sp. TSK-MASC.RSC Adv,2014:4:13045.

19. Shipra das, Surendra Singh, Vinni Sharma, Manohar Lal Soni. Biotechnological applications of industrially important amylase enzyme. Int J. Pharm Bio Sci,2011:2:486-496.
20. Soccol CR, Vandenberghe LPS. Overview of applied solid-state fermentation in Brazil. Biochemical Engineering Journal,2003:13(2-3):205-218.
21. Teixeira EM, Pasquini D, Curvelo AC, Corradini E, Belgacem MN, Dufresne A. Cassava bagasse cellulose nanofibrils reinforced thermoplastic cassava starch. Carbohydrate polymers,2009:78(3):422-431.
22. Vijayaraghavan P, Kalaiyarasi M, Vincent SGP. Cow dung is an ideal fermentation medium for amylase production in solid-state fermentation by Bacillus cereus. J Genet. Eng Biotechnol,2015:13(2):111-117
23. Wu WX, Mabinadji J, Betrand TF, Wu WX. Effect of culture conditions on the production of an extracellular thermostable alpha-amylase from an isolate of Bacillus sp. J Zhejiang Univ Agric Life Sci,2009:25:404-408.