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OPTIMIZATION OF PHYSICAL PARAMETERS FOR PRODUCTION OF AMYLASES AND PROTEASES FROM SOIL BACTERIA

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Abstract–Enzymes are biomolecules present in the cells of living organisms in minute amounts and are capable of speeding up chemical reactions, without being altered and utilized in the reaction. Microbial enzymes are preferred than both plant and animal sources because they are cost effective with respect to production, more predictable, controllable and reliable. These naturally occurring enzymes are quite often not readily available in sufficient quantities for food applications or industrial use. However, by isolating microbial strains that produce the desired enzyme and optimizing the conditions for growth, commercial quantities can be obtained. Our study aimed at isolating and identifying the bacterial strains from soil which produce extracellular proteases and amylases and the optimization of physical conditions for maximum enzyme production.

INTRODUCTION

Extracellular Proteases and Amylases are naturally produced by microorganisms for breaking down proteins and Carbohydrates. The commercial uses of these enzymes are innumerable. The effective catalytic property of these enzymes have promoted their introduction to several industrial products and processes. Enzymes found in nature have been used since ancient times in the production of food products like cheese, beer, wine, vinegar and in manufacturing of commodities such as leather, indigo, linen etc. (Hema and Shiny, 2012; Kolb *et al*., 1996; Oyeleke and Oduwole, 2009). These processes relied either on naturally produced enzymes from microbes or from fruits and vegetables. Microbial enzymes are preferred than both plant and animal sources because they are cost effective with respect to production, are more predictable, controllable and reliable. The majority of currently used industrial enzymes are hydrolytic in nature. Proteases remain the dominant enzymes because of their extensive use in detergent and diary industry. It was seen that protease produced *by Bacillus clausii* SM3 had high capability of removing the blood stains, which indicates its potential in detergent industries. From this study we came to know that the study organism *(Bacillus clausii* SM3) isolated from soil can be used as an effective source for the production of protease enzyme (Oyeleke and Oduwole, 2009). A new strain of *Bacillus* was found to be a potential producer of protease enzyme. Studies on *Bacillus* sp. N- 40 showed that nutritional factors including sources of carbon, nitrogen and metal ions can influence production of protease.

In 1835 amylase was purified first from malt. The amylase family of enzymes is of great significance due to its wide area of application. Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders. Microbial amylases have successfully replaced the chemical hydrolysis of starch in starch-processing industries. They would be potentially useful in the pharmaceutical and fine chemicals industries if enzymes with suitable properties could be prepared. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medicinal and analytical chemistry. Amylases used in industries such as starch, textile, detergent, baking represent second largest group. A lot of work has been done to study the different roles being played by amylases and proteases and their physiological effects. We studied the different physiological parameters like Incubation period,

pH, temperature for the maximum production of amylases and proteases from different bacterial strains isolated from soil.

MATERIALS AND METHODS

Soil samples were collected from the premises of Reva Institute of Science and Management (Pandey *et al*., 2010), Bangalore. Different bacterial colonies were isolated and characterized on the basis of physical and biochemical tests in accordance with "Bergey's Manual of characterization" for micro-organisms. Bacterial colonies grown were characterized on basis of morphology like margin, elevation, pigmentation, texture, appearance, form and their size. After colony characterization the dominant colony was stained to elucidate the morphology and arrangement of the bacterial cells, followed by different staining techniques like gram staining, spore staining and capsule staining.

Screening of identified bacteria for production of protease

Preliminary tests for protease production with identified bacterial colonies were done by using agar cup assay method. Biochemical assays were done as the final confirmatory test.

Cup-assay for protease

The isolated bacterial colonies were streaked on skim milk media for screening of protease activity. Presence of clear zone around the streaking area due to degradation of skim milk showed the activity of proteases.

Table 1. Result of different types of staining

Colony no.	Grams	spore	Capsule
A	+ve	-ve	+ve
B	+ve	+ve	-ve
C	-ve	-ve	-ve
D	+ve	-ve	-ve

Biochemical assay for protease

The bacterial strains found positive with the cup assay were used for biochemical assays. Nutrient broth (NB) was inoculated with *Azotobacter, Bacillus subtilis* and *E.coli* (Fernanda *et al*., 2007; Rao *et al*., 2006). The inoculated culture flasks were incubated on shaker for 24,48 hrs and 60 hrs respectively at 37 °C. Later the culture was centrifuged and supernatant was used for assay. Protease activity was assayed by this method 1 ml of the culture supernatant was mixed thoroughly with 1 ml of 2% casein solution. Mixture (enzyme and substrate) was incubated at 37 °C for 10 min. The reaction was terminated by the addition of 2ml of 0.4M trichloro acetic acid, it was further incubated for 20 min at 37 °C. The incubated solution was filtered through Whatman no:1 filter paper. 1ml from the filtrate with 5ml of 0.4M sodium carbonate and 0.5 ml of 0.5 N folin phenol reagents were added and mixed thoroughly, it was again incubated at 37 °C for 20 min. Optical density (O.D) was measured at 660 nm. Parallel to it a standard BSA graph was also prepared to estimate our enzyme activity. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine in 20 min at 37 °C.

Screening of identified bacteria for production of Amylase: Cup-assay for amylase

The Identified bacterial colony were screened for the amylase production by streaking on starch agar plates (Table 4) and kept for incubation for different time period. The iodine solution was added to

Table 2. Result for the Biochemical tests

Colony no.	Catalyse activity	Oxidase activity	Citrate utilization	N_2 free media	Glucose fermentation	Aerobic/ Anaerobic	Indole Production	Mannitol Fermen-tation	Lactose fermen tation
A	+	+	-	+	Acid Production	Obligate Aerobe	-	-	-
B	-	+	-	-	-do-	Facultative anaerobes	-	-	-
C	+	+	+	-	-	Facultative anaerobe	-	-	-
D	+	-	-	-	-do-	Anaerobe	-	+	-

After completion of biochemical test we confirmed the presence of bacterial strains;
A-*Azotobacter nigricans*, B-*Bacillus subtilis*, C-*E.coli*, D-*Staphylococcus aureus*

observe the presence of clear zone around the streaking area due to the starch hydrolysis, that shows the production of amylase enzyme (Hema and Shiny, 2012 and Jatavathu *et al*., 2011). *Bacillus subtilis, E.coli* and *Azotobacter* were found to be positive for amylase production as well. Apart from them some degree of amylase production was also observed in *Pseudomonas aeruginosa*. As the activity was very low from *pseudomonas aeruginosa*, we did not consider it as candidate for the enzyme production.

Biochemical assay for amylase

Nutrient broth (NB) was prepared and inoculated with bacterial strains (*Bacillus, Azotobacter* and *E.coli, Pseudomonas*) found positive in agar cup assay and were incubated for 48 hours. Culture was centrifuged at 10,000 rpm for 15 minutes. Supernatant was used as source of extra cellular amylase.

Amylase activity was checked by measuring the reducing sugar formed by the enzymatic hydrolysis of soluble starch. The assay mixture containing 1.0 ml of the crude enzyme, 1.0 ml of 0.1 M phosphate buffer (pH 7.0), and 1 ml of 1% soluble starch were added. The reaction mixture was incubated at room temperature for 3 minutes. The amount of reducing sugar released was determined by adding 3, 5 dinitro salicylic acid and boiling for 5 minutes. Absorbance was read at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme that releases one µmol reducing sugar equivalent to glucose per min under the assay condition.

Optimization studies

The optimum temperature, pH, incubation period was determined for production of maximum amount of extracellular amylase and protease from different bacterial strains. To determine the optimum incubation time for production of maximum amount of protease, the test organisms (*Bacillus* and *Azotobacter, E. coli*) were used for cup assay as well as were grown in skim milk media for different incubation period (24,48,62 hours) in an orbital shaker. The contents were centrifuged at 10,000 rpm at 4°C for 15 min and protease activity was checked in the supernatant by biochemical assay. Similar studies were done to optimize the temperature (15 °C, 37 °C, 42 °C, 60 °C and 70 °C) and pH (6.0, 7.0 and 8.0). Both cup assay and biochemical assay was done to find optimum temperature and pH conditions for maximum extracellular enzyme production.

RESULTS

Basic staining and biochemical characterization confirmed the presence of A-*Azotobacter nigricans*, B-*Bacillus subtilis*, C-*E. coli*, D-*Staphylococcus aureus.* Protease activity was prominent in *Bacillus* strain after 48 hours, though activity was also observed in other strains. Important to note was the fact that even after 62 hours *Azotobacter* and *Bacillus* was still having moderate protease activity. Results of biochemical assay for protease confirmed the results

Table 3. Cup assay for protease

Strains	After 24 hrs	After 48 hrs	After 62 hrs
A	-	++	++
B	+	+++	++
C	-	++	+
D	**-**	**-**	**-**

+ = 0.5 to 1.0 cm, ++ = 1.0 to 2.5 cm, +++ = more than 2.5 cm (Diameter of clear zones) *A-Azotobacter nigricans, B-Bacillus subtilis, C- E.coli, D-Staphylococcus aureus*

Table 5. Cup assay for Amylase

Strains	After 24 hrs	After 48 hrs	After 62 hrs
A	-	+	++
B	+	+++	++
C	-	++	-
D	-	-	-

+ = 0.5 to 1.0 cm, ++ = 1.0 to 2.5 cm, +++ = more than 2.5 cm A-*Azotobacter*, B-*Bacillus subtilis*, C-*E.coli*, D-*Staphylococcus aureus*

Table 4. Biochemical assay for protease

Strains	After 24 hrs OD at 660 nm	After 48 hours OD at 660 nm	After 60 hrs OD at 660 nm
A	0.06	0.34	0.30
B	0.2	0.66	0.38
C	0.03	0.50	0.10

A-*Azotobacter*, B-*Bacillus subtilis*, C-*E. coli*

Table 6. Biochemical assay for Amylases

Strain	After 24 hrs OD at 540 bm	After 48 hrs OD at 540 bm	After 62 hrs OD at 540 bm
A	0.05	0.28	0.41
B	0.32	0.60	0.48
C	0.02	0.29	0.05

A-*Azotobacter nigricans*, B-*Bacillus subtilis*, C – *E. coli*
Optimization studies:

of cup assay. The different pH conditions were set to observe the optimum pH conditions for the protease activity from *Azotobacter, E. coli* and *Bacillus*, *Bacillus* was found to be working best at pH

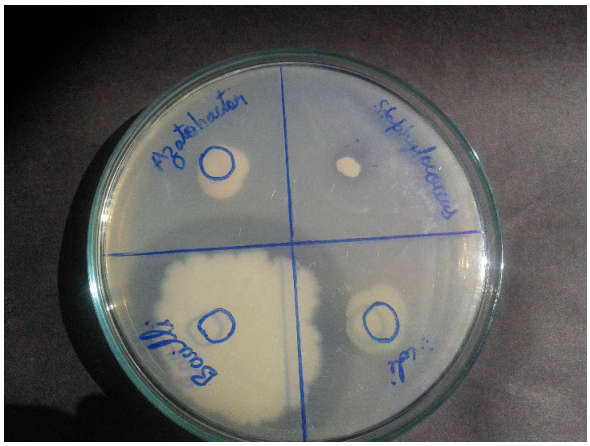

Fig. 1. Cup assay for Proteases

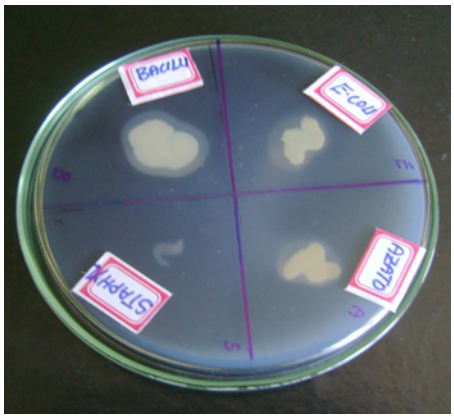

Fig. 2. Cup assay for amylase.

Table 7. Proteases: optimum pH

Strains	pH 6OD at 660	pH 7OD at 660	pH 8OD at 660
A	0.12	0.56	0.36
B	0.28	0.49	0.64
C	**0.31**	**0.60**	**0.41**

A-Azotobacter, B-Bacillus subtilis, C- E.Coli

8 whereas *E.coli* was working best at pH 7. Azotobacter was moderate in its activity at both the pH. The temperature of 42.0 was found to best for the protease activity by all three strains, though activity was observed at 60.0 also. Above and below this temperature the secretion of protease enzyme falls drastically.

Table 8. Proteases: Optimum Temperature

Strains	37 °C	42 °C	60 °C	70 °C
A	0.30	0.42	0.40	0.15
B	0.40	0.57	0.44	0.29
C	**0.41**	**0.54**	**0.38**	**0.21**

A-Azotobacter, B-bacillus Subtilis, C-E.coli

Amylase synthesis and secretion was maximum from *E.coli* strain, pH between 6-7 was found to be optimum for synthesis and secretion of amylase from *E. coli.* Other strains were found to be active for amylase synthesis but their secretions were very low as compared to *E. coli* strain. Temperature of 42 was found to be most suitable for amylase activity, though at 60 also activity was observed.

CONCLUSION

The bacterial colonies were identified on the basis of staining, colony characters and biochemical testing. Four bacterial colonies were identified in accordance with Bergey's Manual of Determinative Bacteriology. *Bacillus subtilis* was found to be actively involved in the extracellular secretion of both amylases and proteases under similar physical conditions of incubation period, temperature and pH. Incubation period of 48 hours was best for *Bacillus subtilis* for amylase as well as for protease production. pH 6.0 was optimum for Amylase production by *Bacillus* whereas pH 8.0 was best for protease production by the same genus. A pH range of 6-8 was required by different bacterial strains for production of amylases and proteases. Optimum temperature for amylase and protease production

Table 9. Amylases : Optimum pH

Strains	pH 6OD - 560 nm	pH 7OD – 560 nm	pH 8OD – 560 nm
A	0.30	0.26	0.28
B	0.79	0.43	0.31
C	0.28	0.25	0.29

A-Azotobacter, B-Bacillus subtilis, C-E. coli

Table 10. Amylases: Optimum Temperature

Strains	37 °COD -560 nm	42 °COD 560 nm	60 °COD 560 nm
A	0.26	0.27	0.40
B	0.43	0.62	0.51
C	0.28	0.36	0.31

A-*Azotobacter, B-Bacillus subtilis, C-E. coli*

was found to be 42 °C by *Bacillus* whereas a high temperature of 60 °C was found suitable for enzyme production by Azotobacter. Though at 42 °C temperature production was maximum but even at temperatures of 60-70 °C amylase and protease production was found to be there.

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