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PRODUCTION AND PURIFICATION OF AMYLASE FROM BACILLUS SPECIES.

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ABSTRACT

Efficient amylolytic strains of Bacillus sp. were isolated and screened. The identification of strains was the result of initial screening based on the hydrolysis of starch ratio. Secondary amylase-based screening in the starch broth medium resulted in the identification of amylolytic strains of Bacillus sp. the selected strain was grown in fermentation media to test for optimization and characterization.

Key words: Amylase, Bacillus, Hydrolysis, Fermentation, Optimization.

INTRODUCTION

Amylases are a group of glycosidic enzymes which hydrolyze α-1,4 starch binds to dextrin and various monomeric products. Microbial amylases are incredibly significant features for bulk development and simple genetic modification **[1].**

Bacterial amylases are considered to be greater constancy, high efficiency and lower production costs. The Bacillus genus is significant producer of many extracellular enzymes including amylases. Bacillus sp. is the best alternative source for commercial development by showing the contribution such as short fermentation cycle, handling is safe, consistency, successful production of the enzyme under stress conditions and ecofriendly features **[2,3].**

The proliferation of microorganisms is due to their diversity, but approximately 96% of microorganisms were not cultures in laboratory conditions. Tracing those microbes will lead to successful achievements in numerous fields of biotechnology. In terms of the applicability of such microbes towards environmental issues, characterizing and isolating them in utterly necessary **[4].** An important role is played by the amylase in biotechnological studies and also placed at important position in the enzyme market in world **[5,6,7,8].**

The wide-ranging application of amylase found in various products such as cosmetics, nutritional, and pharmaceutical processes to increase their significance **[9].**

Amylase derived from number of sources for example, microorganisms, plants and animals **[10].**

The current research was aimed to reporting the characteristics of an unique amylase producing bacteria from rotten fruits, to achieve high enzyme output and greater enzyme activity in optimized conditions.

METHODOLOGY

Sample collection:

Different rotten fruit samples were collected from different location to obtain the desired species **[11]**.

Isolation of amylase producing bacteria from sample:

The bacterial cultures were isolated from samples after serial dilution and spreading. These cultures were shortlisted on the basis of morphological parameters and then converted to pure cultures using streak plate method **[12]**.The cultures were screened on the basis of zone of hydrolysis observed after iodine flood on the cultures containing MSM plates supplemented with 1% starch **[13,20]**.

Estimation of amylase:

The estimation of amylase enzyme was analyzed by DNS assay and Lowry's assay **[14]**.

Identification of bacteria:

For bacterial culture identification, various staining's and biochemical tests were carried out which were based on Bergay's manual **[15]**.

Selection and optimization of the production media:

For enhancing the production and activity of amylase the media selection and optimization were done on the basis of one factor at a time. The best components were selected by observing the bacterial growth and the estimation of amylase **[16]**.

Fermentation and purification of amylase:

The fermentation media was prepared and then incubated for 48 -72 hours after inoculation for the production of amylase **[17]**.

The fermentation is based on shake flask method. Further downstream processing was performed by salt precipitation and dialysis **[18,19,20]**.

RESULTS

Collection of sample:

The samples were collected from various fruit and vegetable shops of Gomtinagar, Lucknow to obtain the desired cultures, as described in below table 1.

Table 1: collected samples

Sample type	Samples
Apple	
Ber	

Capsicum	
Lemon	

Isolation of bacterial culture:

The bacterial cultures were selected on the basis of the primary and secondary screening followed by the isolation of pure culture from the samples, where S2C1 was selected as best on the basis observing the maxim and clear zone of hydrolysis as shown in figure 1,2 &3.

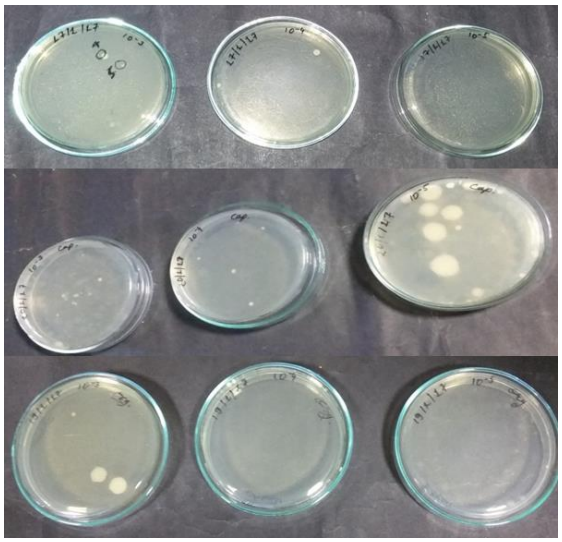

Figure 1: above figure represent serial dilution plates of different samples for bacterial isolation

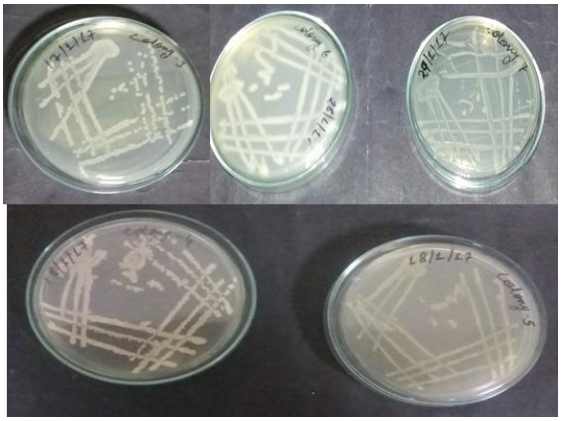

Figure 2: Above figure represent few purified bacterial colonies

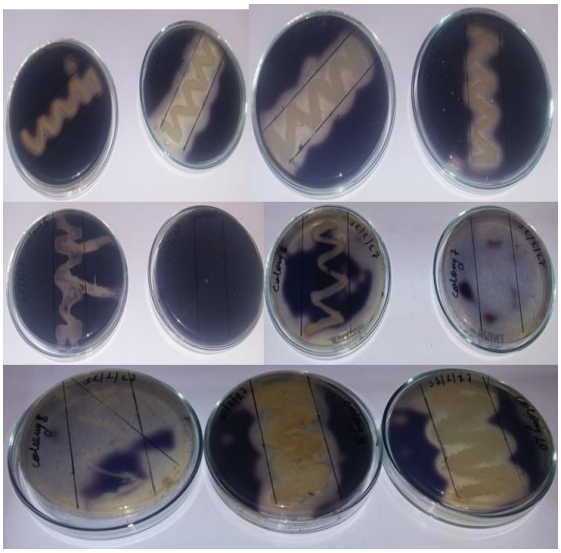

Figure 3: Above figure represent primary and secondary screening of bacterial colonies

Selection of production media:

Table 2: Given table represent the results of DNS Assay of different production media after 48 hours.

PRODUCTION MEDIA	O.D. AT 540nm	CONC. OF EZYME	ENZYME ACTIVITY
Media-1	1.03	1.8	0.0864
Media-2	0.51	0.9	0.0434
Media-3	0.27	0.45	0.0216
Media-4	0.37	0.35	0.0168

Table 3: Given table represent the results of DNS Assay of selected production media with different nitrogen sources

Nitrogen Sources	O.D. AT 540nm	CONC. OF EZYME	ENZYME ACTIVITY
MM-1 (Yeast)	1.65	2.9	0.139
MM-2 (Peptone)	1.63	2.85	0.1368
MM-3 (Tryptone)	1.68	2.9	0.139
MM-4 (ammonium sulphate)	1.45	2.55	0.1224

Table 4: Given table represent the results of DNS Assay of selected production media with different carbon sources

Carbon Sources	O.D. AT 540nm	CONC. OF EZYME	ENZYME ACTIVITY
MM-5 (Dextrose)	1.61	2.8	0.1344
MM-6 (Maltose)	2.00	3.55	0.1704
MM-7 (Mannitol)	0.27	0.9	0.00432
MM-8 (Lactose)	2.00	3.55	0.1704

Table 5: Given table represent the results of DNS Assay of selected production media with different metal ions

Metal ion Sources	O.D. AT 540nm	CONC. OF EZYME	ENZYME ACTIVITY
MM-9 ($FeSO_4$)	0.92	1.6	0.0768
MM-10 ($MgSO_4$)	0.93	1.6	0.0768
MM-11 ($ZnSO_4$)	0.74	1.2	0.0576
MM-12 ($CaCl_2$)	0.61	1.05	0.0504

Fermentation and downstream processing:

Table 6: Given table represent the results of

Enzyme	O.D. AT 680nm	CONC. OF Protein
Crude enzyme	0.96	0.22
Pure enzyme	0.49	0.48

Enzyme	O.D. AT 540nm	CONC. OF EZYME	ENZYME ACTIVITY
Crude enzyme	0.46	0.8	0.033
Pure enzyme	0.62	1.05	0.0504

DNS Assay of crude and pure enzyme.

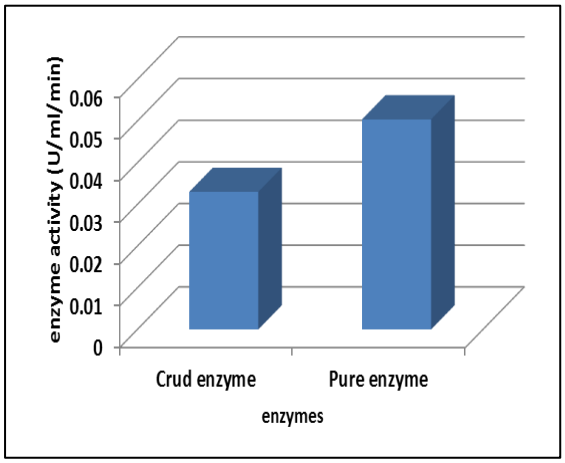

Figure 4 : Given graph represents the enzyme activity of pure and crude enzyme.

Table 7: Given table represent the results of lowery assay of crude & pure enzyme.

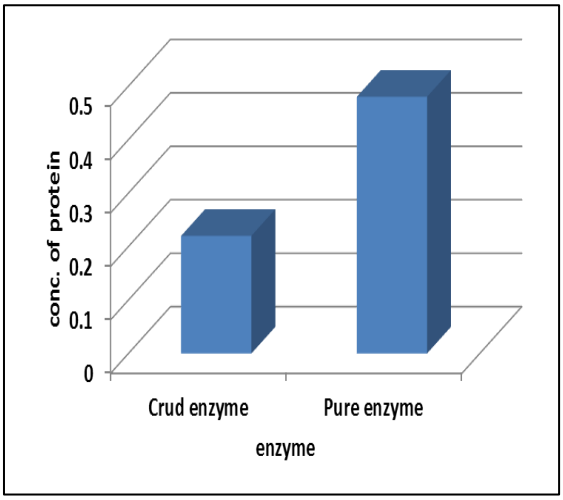

Figure 5: Given graph represents the protein content of pure and crud enzyme

Strain Identification:

Table 7: results of staining and biochemical tests.

Tests	Remarks
Gram Staining	Gram positive, rod shape
Mannitol test	Positive test
MR-VP test	Negative test
Catalase test	Positive
Endospore est	Negative
Mannitol test	Positive
Glucose fermentation test	Positive

DISCUSSION AND CONCLUSION

In our project we have compared the ability of enzyme production from bacterial species isolated from same source. For that purpose we have collected the fruit and vegetable sample from our local market. We have randomly selected apple, orange, ber, lemon, capsicum and kept them for few days so that they get spoiled. Then we have isolated the bacterial and fungal culture by serial dilution method. The serial dilution is done in 0.85% NS solution up to 10^{-6} dilution. Now we have 10 bacterial and 5 fungal colonies from 5 different fruit and vegetable samples.

The bacterial cultures are then purified using quadrant streaking method. Then the colonies are subjected to primary and secondary screening to isolate amylase producing culture. Screening is done on MAM which contain all necessary salt to maintain the viability of all bacterial isolates, with substrate which only promote the growth of desired colony. After 48 hours of incubation they are poured with iodine solution for secondary screening. On basis of that we have selected colony number-3 for optimization.

We have collected the composition of various media used for the amylase production from different research papers. Randomly selected 4 media are prepared and inoculated with colony 3 after autoclaving. These are incubated for 72 hours at 37° C and after each 24 hour DNS Assay is performed to get best incubation period in every media used. On the basis of O.D. at 540nm Media-1 is selected.

After selection the culture is inoculated in NB for the further use. Now the media is optimized for different carbon (dextrose, maltose, lactose, mannitol), nitrogen (yeast, peptone, tryptone, ammonium sulphate), metal ions ($FeSO_4$, $ZnSO_4$, $CaSO_4$, $MgSO_4$) and for the pH (5, 7, 9, 11). The selection is based on the O.D. of DNS Assay at 540nm.

Now the media is optimized for nitrogen sources (yeast, peptone, tryptone and ammonium sulphate) in same concentration. According to 2% N_2 source is used in each test tube and incubated for 24 hours. The best results are found with tryptone.

For carbon sources (dextrose, lactose, mannitol and maltose) in same concentration. According to 1% carbon source is used in each test tube and incubated for 24 hours. The best results are found with maltose.

For metal ion ($ZnSO_4$, $MgSO_4$, $FeSO_4$ and $CaCl_2$) in same concentration. According to 1% metal ions are used in each test tube and incubated for 24 hours. There is no significant effect of metal ion separately in maximum amount.

Now the media is optimized for pH (5, 7, 9 and 11) in same media as selected after optimization. After maintaining pH, test tubes are incubated for 24 hours. The results obtained at each pH are approximately same. So we randomly select pH-7. After the optimization, optimized sources are used for fermentation media and incubated for approximate 72 hours at 37°C. This media is centrifuged to extract crud enzyme. This crud enzyme is precipitated with ammonium sulphate salt and dialyzed and incubated at 4°C in tris buffer to obtain pure enzyme. After doing the DNS Assay and lowery we obtain the enzyme activity of crud enzyme 0.033 and pure enzyme is 0.0504. The specific activities are 6.66 and 9.523 respectively.

In our study we found that *Bacilus megaterium* is best grown in media containing tryptone as N_2 source, maltose as carbon source and pH 7. Under following conditions it gives enzyme activity 0.0504 with specific activity 9.523.

In future we would like to purify the enzyme with the help of HPLC, increase the enzyme concentration and their activity.

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