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STUDIES ON PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ALKALINE AMYLASES FROM BACTERIA (*Bacillus megaterium*)

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ABSTRACT

The Amylases are currently the most essential enzymes utilized in the industry. Alkaline amylases are the ones obtainable in alkaline circumstances. Such enzymes hydrolyze the starch molecule into polymers of short form consisting of a glucose segment such as maltose. This research contains 16 major amylase-producing bacterial isolates collected during primary screening. Secondary screening of these bacteria results in the development of strongly alkaline amylase isolates known as Bacillus megaterium. Such bacterial strains have been found to develop complete alkaline amylase activity at pH 11 in 5.8unit / min / ml. Amylase enzyme was further tested for their biochemical properties under differing temperature, pH, activator impact and inhibitor impact conditions. Tests indicate the enzyme was generated within the 7-11 pH range. The optimal temperature for enzyme development was 37°C at pH 11.

KEYWORDS- Amylase, Starch, Starch hydrolysis, Enzyme activity,

INTRODUCTION

Amylases are among the most essential industrialized enzymes in the meat, processing, fibre, paper, sugars and washing powder industries and have a large variety of applications. α-Amylases (E.C. 3.2.1.1.) are starch degradation enzymes which facilitate the hydrolysis of internal α-1, 4-O-glycosidic bonds in polysaccharides with the preservation of α-anomeric structure in low molecular weight products such as glucose, maltose and maltotriose **[1,2].**

Amylases are the foundational enzyme of starch brerakdown. Since the enzyme arises from other outlets, such as plant species and microorganisms. In industry they are mainly formed from the microbes. They find many uses in production, such as the washing powder, clothes, paper, fruit, and ferment industries. During the screening of bacterial isolates 16 strains were found to produce alkaline amylase (pH 11). Within this study the strain improvement and biochemical characterization of alkaline amylase is listed in the Bacillus megaterium (MJPK 2015 16).

STARCH

Starch is an important resource o in the human diet and is enzymatically and scientifically transformed into a variety of specific products such as hydrolysates, glucose syrup, fructose, maltodextrin derivatives or cyclodextrins found in the food industry **[3-7].** In addition to this, the sugar produced for obtaining ethanol must be fermented. Maize, tapioca, potato, and wheat are the major agricultural source of starch, but weaknesses such as poor tolerance to shear, pyrolysis, and high susceptibility to retro gradation hinder their use in other industries **[8,9].** Starch is currently experiencing growing popularity among carbohydrate polymer because of its utility in various food goods. Starch contributes greatly to the textured properties of several foods and is widely used as a thickener, colloidal stabilizer, gelling agent, bulking agent, and water retaining agent in food and industrial usage. Starch is a polymer of glucose, linked to another by the glycosidic bond. Two forms of glucose from polymer contained in starch: Amylose and amylopectin. Amylose and amylopectin have distinct results and are structured **[9-12].** Amylose is a linear polymer composed of up to 6000 glucose units with α-1,4 amylopectin glycosidic bonds composed of short α-1,4 linked to 10-60 glucose units of the linerar row, and α-1,6 linked to side row 15-45 glucose units.

METHODOLOGY

Sample collection and isolation of bacteria

The soil was sampled from four separate locations (from 0-5 cm depth) of pH 11 namely Lucknow (3) and Jhansi (1). These samples were enriched with raw potatoes in the laboratory and left for 10 days. Dilution was produced from 10^{-1} to 10^{-9} and alkali bacteria were extracted selectively on pH 11 of the Nutrient Agar media. Such bacterial colonies were screened for development of amylase utilizing minimal agar medium with 1 percent starch and isolates developed a strong zone of starch hydrolysis **[13].**

Identification of potent bacterial strain

For the identification of selected culture which was used for further research work, done by gram stainning, endospore staining, manitol fermentation test, MR-VP test, and catalase test **[14].**

Strain improvement

The isolated bacterial strain was subjected to strain improvement with EtBr (Ethidium Bromide), which is supplied at a concentration of 1µg / ml, 2µg / ml, 3µg / ml, 4µg / ml, 5µg / ml and UV (ultra violet) rays of 2min, 4min, 6min, 8min, 10min in duration. In the latter analysis the concentration of EtBr 3µg / ml was found to be the highest for strain development of MJPK 2015 16 strain **[15].**

Growth curve

For bacterial growth curve, the isolated culture was inoculated in 100 ml of sterile nutrient broth and incubated at 37 °C/ 120 rpm in shaker incubator. OD was read at 620 nm after every hours against 5 ml freshly prepared NB media **[16].**

Effect of temperature

For observing the effect of temperature, the selected strain was streaked on the nutrient agar media and was incubated at different temperatures randomly 22°C, 28°C (RT), 37°C and 50°C **[17].**

Effect of pH

The chosen strain was inoculated into four test tubes that had the nutrient broth of specific pH 5, pH 7, pH 9, and pH 11 in order to determine the influence of pH, and incubated at 37 °C, 120rpm for 24hours, Has been tested at 620 nm **[18].**

Media composition and growth conditions (chemical optimization)

Effect of the following on amylase activity was studied: Starch (1.5%), NaCl (0.5%), KH_2PO_4(0.1%) (Alternate sources of carbon (maltose 1%), nitrogen (beef extract0.5%), and metel ion substrate ($FeSO_4$ 0.01%). One unit of amylase activity was defined as the amount of enzyme that released 1 µmol maltose per ml per minute.

Extraction of crude enzyme by shake flask fermentation

Batch Fermentation through the shake flask

A method in which the bacteria are produced in a liquid medium which is actively aerated and agitated in fermenter is often classified as shake flask fermentation. In batch fermentation, bacteria inoculated in known volume of culture medium for a given period of time. , and then the cell mass is removed from the liquid before further processing. In this experiment 20 µl of selected bacteria were inoculated in 100 ml of optimized fermented media and incubated at 37^0c, 120 rpm for 1 week.

Partial purification of amylase (salt precipitation)

The crude enzyme is purified by 40% ammonium sulphate precipitation method

Dialysis

Dialysis was performed to purify the enzyme from the contamination like traces of salt present in the crude enzyme**.**

Effect of pH and temperature for enzymatic activity

Partially purified enzyme was incubated with the different pH (pH 5.0, 7.0, 9.0 and 11.0). Enzyme activity at different temperatures was determined by incubation at 22°C, 28°C, 37°C and 50°C.

Effect of metal ions and chelator

The Metal ions $ZnSO_4.7H_2O$, $CaCl_2$, $MgSO4.7H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $CaCl2.6H_2O$, $MgSO_4.H_2O$ and chelator EDTA were evaluated for effect on amylase activity.

RESULTS

Isolation and purification of bacterial strains

Sixteen different isolates were selected from mixed culture plates obtained after serial dilution of bacteria, the colony were differentiated on the basis of colony morphology and named as mjpk2015 01, mjpk2015 02, mjpk 201503, mjpk2015 04,…to mjpk2015 16. All the sixteen bacterial strain were streaked on nutrient agar media plates by disquadrant streaking manner.

Screening of purified bacterial strain for amylase production

All the sixteen isolates were screened for production of Amylase by starch-iodine test on minimal agar medium with 1% starch, result of the same can be seen below in **Table 1.** The isolate MJPK2015 16 showing maximum zone of starch hydrolysis was stained with gram's staining procedure and was found to be gram positive rod shape in chain.

Table 1. Screening of purified bacterial strain for amylase production.

Bacterial strain	Primary screening	Secondary screening
MJPK2015 01	+	-
MJPK2015 02	-	-
MJPK2015 03	-	-
MJPK2015 04	++	+
MJPK2015 05	+	-
MJPK2015 06	-	-
MJPK2015 07	-	-
MJPK2015 08	+++	++
MJPK2015 09	++	-
MJPK2015 10	-	-
MJPK2015 11	++	-
MJPK2015 12	+++	++
MJPK2015 13	-	-
MJPK2015 14	-	-
MJPK2015 15	-	-
MJPK2015 16	+++	+++

(-) No Hydrolysis, (+) Slight Hydrolysis, (++) Moderate Hydrolysis, (+++) Intense Hydrolysis.

Strain improvement of selected bacterial strain

Strain improvement of selected strain was done by UV radiation and EtBr (Ethidium Bromide, treatment to cause the mutation in their genome and found to be of some beneficial character. EtBr (3µg/ml) concentration was found to be a good strain improvement career.

Identification of selected strain

All of the Biochemical test which were performed for identification was positive but only VP test was found to be negative. The strain was identified as ***Bacillus megaterium.***

Physical optimization of mjpk2015 16

The physical optimization includes effect of pH, temperatures and growth curve of selected MJPK2015 16 strain.

EFFECT OF pH

Optimum pH for the growth of MJPK2015 16 was determined and it was found that the isolate grows maximally at pH 7 and 11, thus the production media was maintained at the 11 pH.**Figure 1** below shows result of effect of pH.

Effect of temperature

For studying the best suitable temperature for the growth of the isolate, it was streaked on nutrient agar media plate and growth was quantified based on growth in the plate and 37°C temperature was found to be a good for bacterial growth. **Table 2** below shows the result of temperature.

Growth curve

Figure 2 below shows the growth curve of the isolate MJPK2015 07, it can be seen that the strain was reached to stationary phase between 2nd to 3rd day after incubation.

Effect of optimized production media (chemical optimization)

According to requirements of the selected culture for the better production of secondary metabolites, provided the best sources for the media optimization which are as follows

Starch (1.5%), NaCl (0.5%), KH_2Po_4 (0.1%) Alternate sources of carbon (maltose 1%), Nitrogen source, (Beef extract0.5%), and metel ion substrate (FeSo4 0.01%)

Table 3 shows the result below.

Flask fermentation and salt precipitation

Submerged Fermentation

Also known as shake flask fermentation is a process in which the organisms are grown in liquid medium which is vigoursly aerated and agitated in fermenter. The 95 ml of fermented media of bacterial culture was produce and this was subjected to salt precipitation. In the amout according to 472.2gm in 1000 ml.

Dialysis

Dialysis was performed to purify the enzyme from the contamination like traces of salt present in the crude enzyme. In the picture below, the dialysis bags suspended in the tris buffer. The bags filled with off white fluid in the salt precipitated enzyme obtained from submerged fermentation.

Characterization of purified enzyme

Effect of temperature and pH

The selected purified enzyme was reacted with 1% of 0.5 ml starch substrate and incubated at different temperature for enzyme substrate interaction. And different pH of 0.5 ml, 1% starch and incubated at room temperature. After DNS assay we found the best temperature for the Amylase production was 28°C and 37°C and pH was 7, 9 and 11. The results are shown below in **Table 4** and **Figure NO 3.**

Effect of activators

Activators increased the activity of the enzymes the activators are $MgCl_2$ & $CaCl_2$.

Effect of inhibitors

Inhibitors decrease the activity of the enzyme they are SDS & EDTA.

Table no 2. Effect of temperature

Temperature	Growth	Remark
22°C	No growth	-
28°C	Moderate growth	++
37°C	Intense growth	+++
50°	Slight growth	+

Table 3.Effect of optimized production media.

Production media source	Quantity
Starch	1.5%
NaCl	0.5%
KH_2PO_4	0.1%
Beef extract	0.5%
Maltose	1%
FeSo4	0.01%

Table 4. Effect of Temperature on the Purified Enzyme

Temp.	O.D. (540nm)	Maltose released (mg/ml)	Enzyme activity (unit/ml/min)
22ºC	0.52	0.40	0.0192
28ºC	0.48	0.36	0.0172
37ºC	0.48	0.36	0.0172
50ºC	0.47	0.355	0.01704

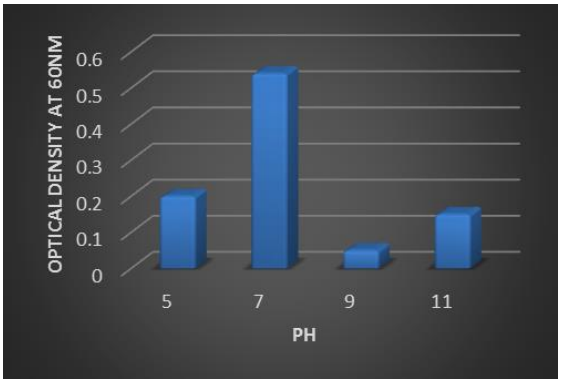

Figure 1. Effect of pH

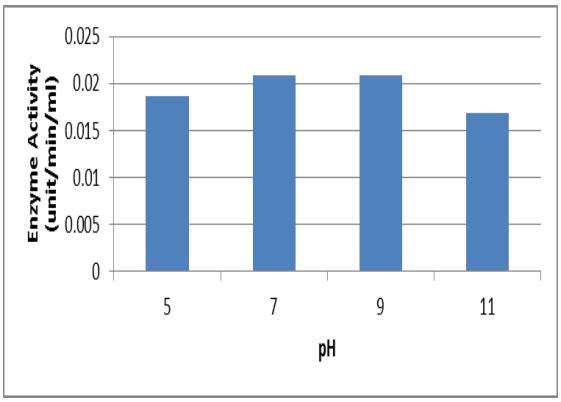

Figure 2. Effect of pH

DISCUSSION

Bacterial was isolated from the soil by serial dilution and agar plating methods as earlier done by **Khan, J.A. 2011** Isolates were further purified which were named as **MJPK2015 01-MJPK2015 16.**

Screening of purified culture was done on MAM (Minimal agar medium) supplement with 1% starch the culture growing in MAM were flooded with Iodine solution and the zone of hydrolysis were obtained in the plate showing starch Hydrolysis similar method has been used earlier by **Suman, S. and Ramesh, K., 2010** in order to screen the microorganisms for amylase production.

Strain improvement was done by UV and EtBr to know the best culture for amylase production.

The bacterial species was identified by the help of various physical characteristic, staining and biochemical activities as done earlier by **Oyeleke, S.B. *et al.,* 2010.**

Submerged fermentation for production of amylase was done earlier by **Riaz, N. *et al.,* 2003.** For amylase production in solid state fermentation the wheat bran is used as a substrate as earlier done by **Saxena, R. *et al.,* 2011.**

Partial purification of the crude amylase was done by ammonium sulphate precipitation & dialysis similar techniques have been used earlier by **Yandri, *et al.,* 2010.**

The reducing sugar were measured by adding DNS reagent, using maltose as standard and the total enzyme activity of purified enzyme was calculated out to be.96 mg/ml and 0.76 mg/ml Previously the enzyme activity was 1.338 U/ml/min by **Aiyer, P. V. D., 2004.**

Protein concentration was measured by Lowery's method **(Lowery. *et al.,* 1951).** Using Bovine serum albumin as standard and the amount of protein in purified sample was calculated out to be 1.47 mg/ml and 1.78 mg/ml for Bacteria and fungus respectively previously the conc. of protein was 10 mg/ml by **Niaz, M. *et al.,* 2010.**

The purified enzyme was characterized for the effect of temperature, pH, activator and inhibitors, 37°C was found to be optimum temperature, pH 9 and 11, earlier they have been characterized by **Kubrak, O.I. *et al.,* 2010** and **Shrivastava, R.A.K., 1987**

CONCLUSION

Finally based on the above study it can be concluded that bacterial species can be a good source for the production of enzyme amylase being used industries.

Amylase purified here was found to be stable in a pH range of 7 to 9 and temperature range of 28 to 37°C. The activity found to be enhanced under the influence of cations such as Ca2+, Mg2+ and retarded under the influence of anions such as EDTA and SDS. The activity of the Amylases purified here is comparable to the activity of amylase purified earlier by various researchers.

The molecular weight was determined by SDS-PAGE and Bands was observed after staining and destaining procedure given indications of purity of amylases. Further work include further purification of the enzyme in order to attain higher specific activity. The purification has to be carried out with further purification process include chromatography technique such as affinity chromatography, ion exchange chromatography and HPLC.

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