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Production of thermostable amylase by the gram negative soil bacterium BLY01

SOMA PAL SAHA*, **SATAKHI HAZRA, ANINDITA HALDER, SHABNAM JAHAN ANSARI, MOUSAM ROY, MAYUKH PAUL AND RINI ROY**

Department of Microbiology, Maulana Azad College, 8, Rafi Ahmed Kidwai Road, Kolkata 700013, W. B., India

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Amylase is chemically a glycoside hydrolase which acts on α-D-1, 4-glycosidic bonds of starch and related carbohydrate. It has immense importance in food and textile industry. Altogether 15 microorganisms have been isolated from soil samples of different parts of West Bengal through enrichment followed by streaking on starch agar plate. The efficiency of the starch degradation of the strains has been primarily determined by iodine assay followed by chemical assay using di-nitrosalicylic acid as reagent. A potent gram negative bacterial isolate designated as strain BLY01 showed the capability of amylase production as high as 56 units/ml (1 unit=10 microgram of reducing sugar formed per ml per minute) and its specific activity was 1.81 mg sugar/min per mg of protein/ml after 24 h of its growth under shake flask condition at 37°C. The K_m value of the enzyme is 0.217 mg of starch. The pH and temperature optima of the enzyme were recorded as 8.0 and 45°C. However, the enzyme showed to retain 25% of its activity even after the heat treatment at 70°C for 10 min. To make the yield of the enzyme cost-effective a variety of non-conventional natural starchy materials were tested and best result was obtained with rotten potato extract.

Keywords: Starch degradation, amylase, thermostable enzyme, *Pseudomonas*

INTRODUCTION

Application of starch in food, bakery, and brewery, confectionaries including fermentation, textile pharmaceutical, detergent and paper industries requires modification of starch. It could be accomplished either by acid hydrolysis or by enzyme treatment. Amylases can be obtained from plant, animals and microbes (Kathiresan and Manivannan, 2006). However, the enzymes from bacterial and fungal source are now considerably exploited in commercial sectors due to their biochemical diversities and actions (Pandey *et al.*, 2000). In the present day biotechnology, approximately 25% of the enzyme market is dominated by amylases (Sindhu *et al.*, 1997).

Amylolytic enzymes are categorized into exo-acting, endo-acting and debranching enzymes. The enzyme, α-amylase (E.C 3.2.1.1), one of the important amylolytic enzymes, is an endo-acting enzyme which catalyses the hydrolysis of D-(1, 4) glycosidic linkages in starch components and related carbohydrates. There are a vast number of reports on starch degrading microorganisms of diverse group and their α-amylase activity (Gupta *et al.*, 2003; Pal Saha *et al.*, 2005; Bose *et al.*, 2009). As it is desirable that á-amylase should be active at high temperatures of liquefaction (70ºC to 90ºC) to economize a variety of commercial process there has been a need for thermophilic or thermostable á-amylases (Crabb and Miotchison, 1997, Sarikaya *et al.*, 2000, Vaseekaran *et al.*, 2010). Considering the above facts, the study has been carried out to isolate a thermostable á-amylase producing strain from starch enriched natural samples and characterization of the organism and the crude enzyme produced by it.

*Corresponding author: Email:spalsaha@gmail.com

MATERIALS AND METHODS

Microorganisms

Wild starch degrading microorganisms were iso-

lated from soil containing waste of a potato cold storage, Bally of Howrah district, West Bengal, India following enrichment, serial dilution and pour plate techniques. Starch broth medium consist of starch- 20g, peptone -5g, beef extract- 3g and distilled water -1000 ml with a pH of 7.0 was used for enrichment culture and the same solid medium with agar 2% (w/v) was used for plating. Organisms on the agar slopes were stored under refrigerated condition at 4°C.

Detection of microbial amylase activity

Duplicate set of plates with growing organisms was flooded with fresh 0.01 (N) iodine in 3% KI solution and organism having starch degrading ability showed a clear halo zone surrounding its colony. Such type of organisms was primarily noted as amylase positive strains. These strains again streaked on fresh starch agar plate and treated with iodine for secondary screening and final screening subsequently.

Characterization of the potent isolate

The selected organism was characterized microscopically and biochemically. Dimension, shape, gram reaction and motility of the organism were determined using light microscope. Utilization pattern of different conventional and non-conventional carbon substrates by the organism were tested on solid agar plates containing the medium same as isolation medium except the sole carbon source. Immobilized calcium phosphate (0.1%, w/v) was added to the agar medium for determination of phosphatase enzyme activity of the organism and it was confirmed by *p*-nitrophenyl phosphate degradation by the culture filtrate of the strain. Casein agar medium and urea broth were used to determine the protease and urease activity of the strain respectively. The catalase activity was tested using hydrogen peroxide on fresh starch agar slant of the strain. Optimum temperature and pH required for growth was recorded using starch broth medium under shake flask condition in a BOD shaker incubator. The antibiotic sensitivity of the organism was tested using twenty three different antibiotics as octadiscs, Himedia.

Enzyme assay

The potent amylase enzyme producing organism was detected following chemical estimation of enzyme. The reducing sugar produced in starch buffer solution (0.1%, w/v) by the crude enzyme present in culture filtrate of the organism grown in starch broth medium was assayed using di-nitrosalicylic acid reagent (Lorenz, 1959). Activity of the enzyme produced by the potent strain was measured in units (U). One unit of α-amylase activity is defined as the amount of enzyme that releases 10µg of reducing sugar per ml per min from soluble starch at pH 8.0 and at 45°C.

Effect of pH and temperature on α amylase activity

Activity of enzyme produced by the organism under shake flask condition was carried out at various pH ranging from 6.0 to 9.0 and the thermal stability of the enzyme was studied at pH 7.5 and at temperature ranging from 30°C to 90°C without additives to evaluate the effects.

Enzyme Kinetics

The specific activity and the kinetics of enzyme were measured. The specific activity of á-amylase produced in broth culture was determined by taking maximum activity of enzyme and cellular protein of the isolate as described by Lowry *et al.* (1951). K_m and V_{max} values of enzyme produced were calculated at 45°C and at pH 8.0 by plotting Lineweaver-Burk plot as mentioned by Lehninger *et al.* (1993).

Use of non conventional substrate

Water soluble fraction of powdered grains of corn, oat, rice, wheat and potato extract, at 2% (w/v) level were tested individually as the sole substrate of carbon instead of conventional soluble starch. The % efficiency of starch hydrolysis and enzyme synthesis (unit/ml) has been determined in agar and broth medium respectively.

RESULT AND DISCUSSION

Isolation of α amylase producing bacteria

Four different soil samples were collected from nearby area of potato cold storage drainage system of Howrah and Kolkata were taken for enrichment culture in starch broth medium for 48h under shake flask condition (160 rpm) at RT. The

Table1 : Characterizations of starch degrading isolate BLY01

	Characterization of bacteria	Result
Cultural characteristics	Colony morphology on starch agar	Small, round, regular, pale yellow, fast growing, non fluorescent.
	Growth temperature range	32^0C - 40^0C
	Growth pH range	7.0-9.0
Microscopic character	Gram staining	Gram negative, small rod
	Spore staining	Non-spore former
	Capsule staining	Non-capsulated
	Motility	Motile
	Dimension	1.2µm X 0.3µm
Biochemical characters	Carbohydrate utilizable	Dextrose, lactose, maltose, and starch
		Arabinose, xylose
	Carbohydrate non-utilizable	
	Acid and gas production in lactose	Negative
	broth	Negative
	Indole	Negative
	Methyl red	Positive
	Voges Proskauer	Positive
	Citrate Utilization	Positive
	Catalase	Positive
	Urease	Positive
	Gelatin hydrolysis	Positive
	Starch hydrolysis	Positive
	Hydrogen sulfide	Negative
	Mobilization of rock phosphate	positive

Table 2 : Antibiotic sensitivity of the isolate BLY01

Sensitive to	Resistant to
Ampicillin	Amphotericin
Azithromycin	Fluconazole
Bacitracin	Netocondazole
Ceftazidime	Iraconazole
Cefuroxine	Nystatin
Chloramphenicol	
Doxycycline HCl	
Erythromycin	
Gatefloxacin	
Lenofloxacin	
Nalidixic acid	
Norfloxacin	
Novofloxacin	
Penicillin	
Pipemidic acid	
Tetracyclin	
Trimoxazole	
Vancomycin	

culture was processed by serial dilution followed by spread plate method for isolation of starch degrading bacteria. A total of 13 different bacterial strains were isolated on the basis of their colony morphology. Following primary and secondary screening on starch agar plate using iodine solution as reagent, a potent isolate designated as BLY01 had been selected considering the diameter of halo zone surrounding the colony after 24h of growth and the starch hydrolysis efficiency

Table 3 : Synthesis of amylase from non-conventional substrates

Substrate# (water soluble fraction)	% efficiency of starch* degradation	Enzyme** unit/ml
Corn	23.43	25.5
Oat	36.0	30.8
Potato	22.72	23.5
Rice	20.58	16.8
wheat	42.85	38.66

1 unit of amylase =10 microgram of reducing sugar formed per ml per minute

The powdered grains (0.2 gm) suspended in 10 ml of hot water , filtered and then filtrate was taken to add to the medium as the sole source of carbon.

*The % efficiency of starch degradation had been determined after 24h of organism's growth on plate containing the solid medium with non-conventional substrate.

** The amylase enzyme was assayed in the culture filtrate after 24h of organism's growth in broth under shake flask condition.

(Fig.1) of the organism was calculated (46%) following the formula,

$$\% \text{ hydrolysis efficiency} = \frac{(x-y)}{y} X100$$

Where, x = breadth of the halo zone (in mm) surrounding the organisms growth y = breadth of the

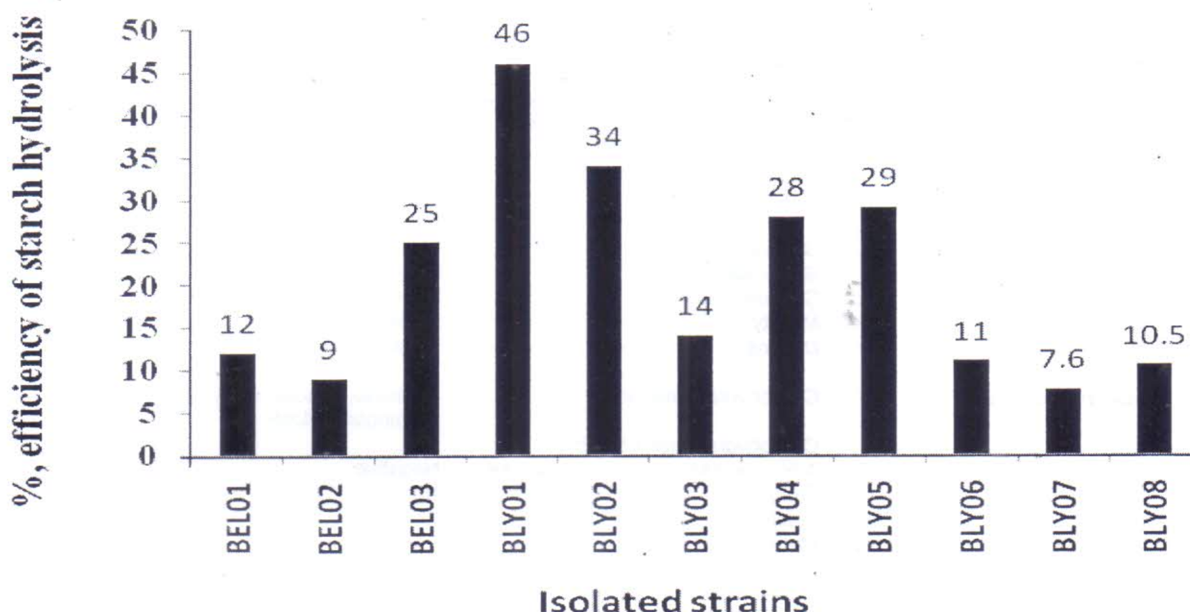

Fig.1. Screening and selection of potent organism on the basis of efficiency (%), of starch hydrolysis by the isolated strains.

The selected organisms were grown on starch agar medium for 24h at 37°C. The colony dimension and the diameter of surrounding halo zone of starch degradation were measured and the % efficiency of starch degradation was calculated accordingly.

growth zone of organism (in mm) .

Characterization of the potent isolate

The bacterial isolate BLY01 was characterized on the basis of cultural characteristics, microscopic appearance and biochemical tests. Considering the entire test results (Table 1) and following the Bergey's Manual of Systematic Bacteriology (second edition) and Bergey's Manual of Determinative Bacteriology (ninth edition) the isolate was identified as a strain of *Pseudomonas* sp. The antibiotic sensitivity of the organism against 23 different antibiotics was also been tested (Table 2) and the strain was found to be sensitive to 18 different antibacterial antibiotics. A good number of soil borne strains of *P. amylodermosa* producing extracellular isoamylase have been recorded as non pathogenic and the enzyme has also been claimed as non-toxic (Harada *et al.*, 1968; Lai *et al.*, 1998).

Utilization of starch and production of amylase

Time course of growth and production of extracellular amylase by the potent strain BLY01 using starch at a concentration of 10 mg/ml as sole source of carbon had been determined (Fig.2A, B and D). The accumulation of reducing sugar in the medium increased consistently (Fig 2C) along with growth up to 24h of the incubation period and thereby utilized by the cells. A slow decline in the amylase production had been depicted in the Fig. 2D which may be correlated with the presence of a negligible amount of residual starch in medium (Fig.2B). The maximum amylase production (56.5 unit/ml) was shown by the stationary phase cells of the strain and the pH of the medium changed from initial neutral point to pH 8.0 at late stationary phase (Fig. 2F) and remained constant throughout the growth period. Specific activity of the crude enzyme had been determined (Fig.2 E) and is defined by the product formed (micromole of reducing sugar) per unit time per amount of protein. Specific activity attained its maxima (1.81 μmol/min/mg of protein) at 20h of growth (growth maxima) before the attainment of peak values of amylase production.

Effect of pH and temperature

Activity of microbial enzymes had been affected by the changes of pH and temperature of the reaction environment. In this study amylase from BLY01 cells did not show any activity below the pH 6.5 and maximum activity was found at pH 8.0 (Fig.3A). More than 73% of the activity of enzyme persists at pH 9.0 of the reaction mixture. The re-

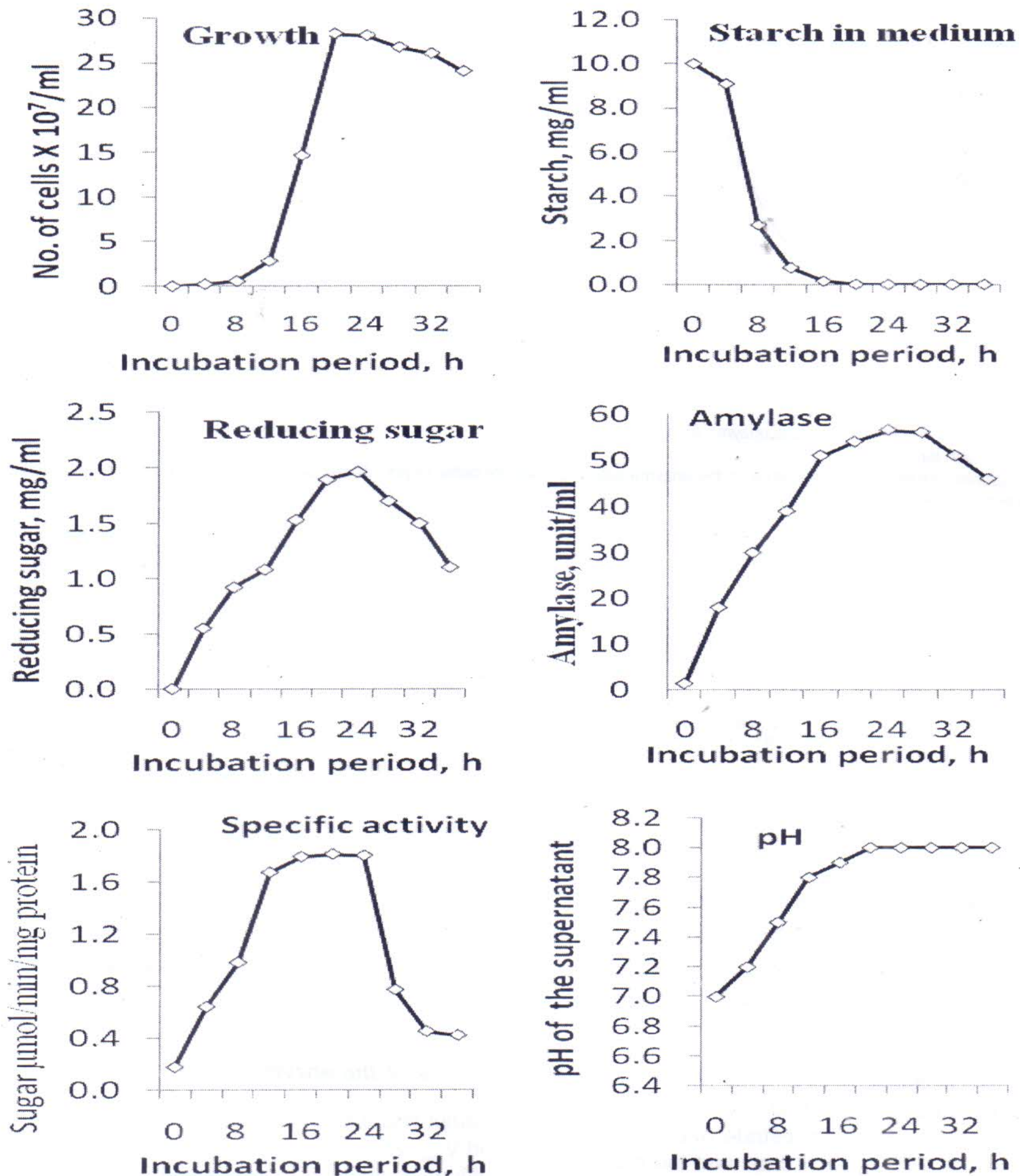

Fig. 2 Time course of growth (A), utilization of starch (B), formation of reducing sugar (C), production of amylase (D), specific activity of the enzyme (E) and changes in pH of the medium during growth (F).
BLY01 cells were grown in starch broth at 37°C for 36h under shake flask condition at 140 rpm. 1 unit of amylase =10 microgram of reducing sugar formed per ml per minute.

sults derived from temperature versus enzyme activity (Fig. 3B) showed that the optimum temperature for the reaction is 45°C and more than 72% activity of the enzyme remained when reaction was performed at 60°C.

Thermal stability of enzyme

Thermal stability of the crude enzyme had been justified after heat treatment of the cell free culture filtrate of BLY01 strain. The filtrate was treated at

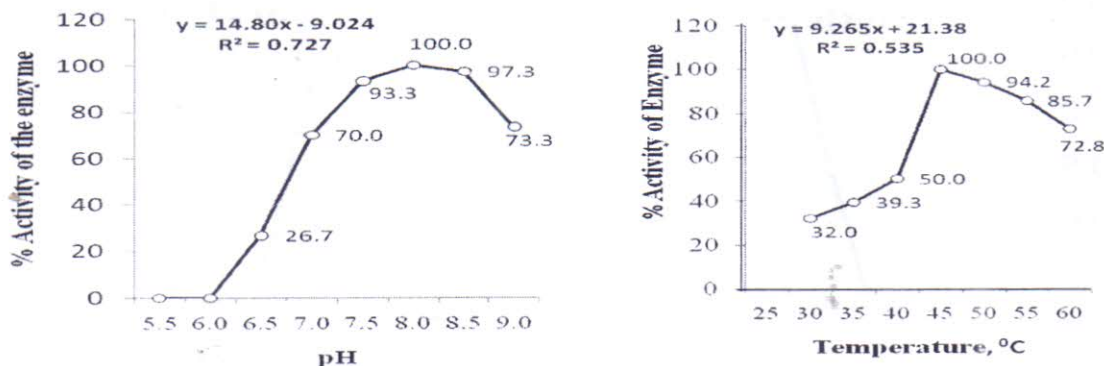

Fig.3 Effect of pH and temperature on the activity of amylase synthesized by BLY01 cells.

BLY01 cells were grown in starch broth for 24h under shake flask condition at 140 rpm and the culture filtrate was taken as crude enzyme.
To determine the effect of pH on the enzyme activity, acetate buffer (pH 5.5 and 6.0) and phosphate buffer (6.5-9.0) were used. In each case reaction temp. was 45°C.
To determine the effect of temperature on the enzyme activity phosphate buffer of pH 7.5 was used at the reaction temperature ranged between 25°C -60°C.

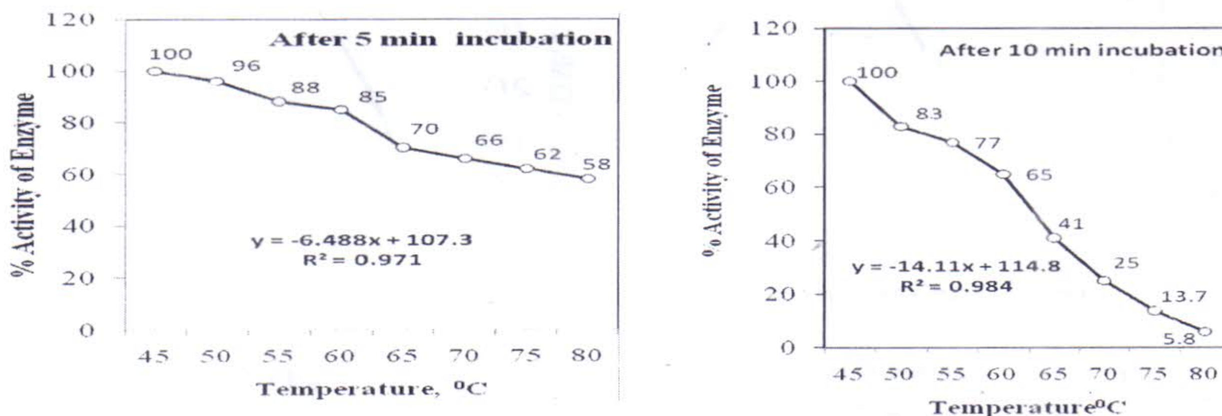

Fig.4. Thermal stability of crude á- amylase isolated from BLY01

BLY01 cells were grown in starch broth for 24h under shake flask condition at 140rpm and the culture filtrate was taken as crude enzyme.
After incubation at different temperature in a hot water bath for 5min and 10 min. respectively, the crude amylase was taken for enzyme assay. The reaction temperature in each case was maintained at 45°C in phosphate buffer of pH 8.0.

different of temperatures, from 45°C to 80°C with 5°C intervals for the period of 5 min. and 10 min., however, the enzyme-substrate reaction was performed at 45°C in each case and the results had been represented in Fig. 4A and 4B. The enzyme produced by the strain showed maximum efficiency at 45°C and it was considered as the 100% activity limit. The percent activity of enzyme declined more rapidly when it was treated for 10 min duration than that of 5 min. period at every temperature point. About 58% and 5.8% activity remained in the crude sample when it was heated at 80°C for 5 and 10 min., respectively. This study indicates that the enzyme produced by the strain is a thermostable enzyme.

Kinetics of the enzyme

Attempt had also been made to determine the K_m and V_{max} values of the amylase produced in batch cultivation of BLY01 cells (Fig.5). The enzyme showed at K_m of 0.2 mg of starch per ml and a V_{max} of 3 X 10^3 OD per min. and it was calculated as 11.65 millimole of glucose per min per ml at 45°C, pH 8.0. The lower K_m value of this thermostable enzyme defined the higher affinity of the enzyme to the substrate.

Utilization of nonconventional substrate for amylase synthesis

The organism BLY01 had shown the ability to grow

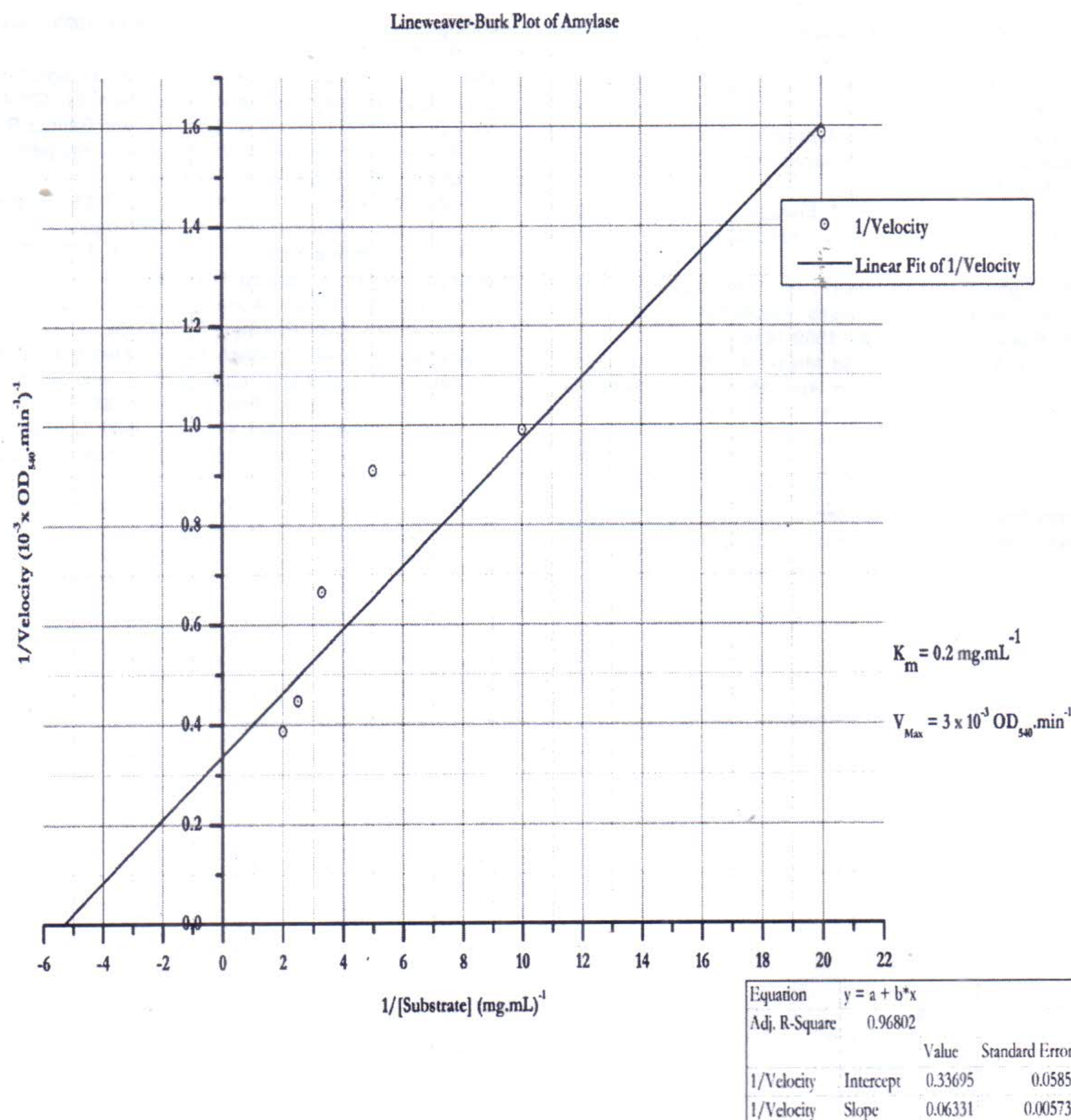

Fig.5. Determination of K_m and V_{max} of the crude amylase of BLY01.

BLY01 cells were grown in starch broth for 24h under shake flask condition at 140rpm and the culture filtrate was taken as crude enzyme. Soluble starch (Merck) as buffer solution was used as substrate at different concentration. The reaction temperature in each case was maintained at 45°C in phosphate buffer of pH 8.0.

on the plates containing different types of non-conventional crude starch source extracted from corn, oat, rice and wheat and potato. The values for starch degradation efficiency and the amylase (unit/ml) synthesis by the organism corresponded in each case (Table 3). The growth, efficiency for starch degradation and amylase activity were found maximum using wheat starch compared to other starch sources used and this might be due to the presence of higher soluble fraction of starch and the other growth promoting factors present in wheat.

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