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Production of extra cellular amylase from waste starches by *Pseudomonas stutzeri*

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A newly isolated strain of *Pseudomonas stutzeri* MTCC 8741 could synthesize extra cellular amylase in presence of various waste starches of which pulse powder showed the best result. The highest production of enzyme was achieved at 24th hour of growth. The enzyme synthesis was enhanced in presence of Ca^{2+} and K^{+} ions but remained unaffected in presence of toxic metal ions like As3+, Pb^{3+} and Li^{3+} and natural antimicrobial agents. The strain showed resistance against marcolide and replication blocking antibiotics.

Key words: Amylase, *Pseudomonas stutzeri,* waste starch antibiotic resistance.

INTRODUCTION

Starch being the most abundant polysaccharide in nature is an easily accessible source of energy. Bioconversion of starch is accomplished by enzymes, collectively called amylase, of which α-amylase hydrolyses the α-1, 4-glucosidic linkages of amylose and amylopectin of starch generally in an endo fashion. This enzyme has extensive commercial applications in starch liquefaction, brewing, in textile industries, and in paper and detergent manufacturing processes. Although a number of microorganisms are known for their α-amylolytic activities, extensive research is going on for new α-amylolytic strains that can utilize waste starchy materials to synthesize the enzyme even in presence of toxic metals and other bactericidal agents.

The present work is a preliminary report of isolation and characterization of an extra cellular amylolytic bacterial strain and evaluation of its potentiality to utilize waste starches in presence of natural and chemical inhibitors.

MATERIALS AND METHODS

In a program of isolation of amylolytic microorganisms, a strain was isolated from starchy effluent rich soil of West Bengal, India. The bacterial strain was identified by its morphological, physiological and biochemical characteristics, and was identified by the criteria of Bergy's Manual of Systematic Bacteriology and later confirmed by and deposited to Institute of Microbial Technology (IMTECH), Chandigarh, India.

The strain was cultivated in 100 ml Erlenmeyer flask containing a basal medium (BM) containing (g/l) : peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4$, $7H_2O$, 0.1 and starch 0.5 (pH-7.0) for 24 hrs. The bacterial growth was measured turbidometrically at 660 nm. For enzyme assay the culture broth was centrifuged at 10,000 g for 10 minute and the supernatant was used as crude enzyme. The amylase activity was measured by incubating the assay mixture (1ml) containing equal volume of properly diluted enzyme and 1% (w/v) soluble potato starch (Merck) in 50 mM phosphate buffer (pH-7) at 60°C for 10 min. The reducing sugar was measured spectrophotometrically at 540 nm using glucose as standard, (Bernfeld, 1955). One unit of amylase activity was defined as the amount of enzyme which catalyzes the liberation of 1μ mole of glucose/min. ml. under optimal conditions. For *in situ* detection of extra cellular amylolytic activities, starch plates with bacterial colonies were flooded with iodine solution (0.2 g/l^{-1} and 2.0 g/l^{-1}) (Ray, 2001). Metal ions and amino acids (100mM), antibiotics (100 μg/ml) and all analytical chemicals used in the experiment were purchased from Sigma (USA) and Merck (Germany). Aqueous extract of the plant materials from dried

pulverized leaves of *Adhatoda vasika, Azadirachta indica, Osimum sanctum;* rhizomes of *Curcuma longa,* stem of *Allium sativum, Zingiber officinale* were prepared (Sengupta *et al.,* 2004) and used at a concentrain of 0.5% (w/v) in the cultivation medium. All the biochemical and physiological tests including the antibiotic sensitivity tests were performed according to Cappuciano and Sherman (1992) and Benson, (1988).

RESULTS AND DISCUSSIONS

The strain was found to be a small, aerobic, gram negative, pigment producing and motile non-fluorescent rod. The strain was positive for catalase, nitrate reduction, cytochrome oxidase, arginine dihydrolase and oxidative fermentation tests but showed negative results in indole tests, methyl red test, Voges Proskauer test, lysine decarboxylase and ornithine decarboxylase tests. It was able to produce acid from arabinose, fructose, galactose, melibiose, raffinose, rhamnose and sucrose. The preferred pH and temperature range for the growth of the strain was 5-11 and 15° -40°C respectively. It could tolerate only 2-4.5% of NaCl in culture media. From these criteria, the strain was indentified and later confirmed by IMTECH as *Pseudomonas stutzeri* MTCC 8741.

Table 1. Effects of various waste starches on the growth and amylase production by *Pseudomonas stutzeri* MTCC 8741

Starch type	Growth A_{660}	Amylase activity (U/ml)
Starch(Sigma)	1.0	40.3
Pulse powder	0.95	39
Flour	0.89	34
Wheat powder	0.87	33.2
Arrow root	0.87	31.9
Corn flour	0.87	30.5
Rice dust	0.8	28.7
Potato	0.9	19
Rice husk	0.6	18
Water chest nut	0.6	6.2

The amylolytic activity of the strain was indicated by the clear zone of hydrolysis around bacterial colony on starch iodide plate. The kinetics of bacterial growth, enzyme production indicated that the present strain growing in optimized fermentation medium at 28°C and pH 7.0 achieved a maximum level of amylase production within 24 hours of cultivation with a fall of initial pH from 7 to 6. Further decline in pH to 5.0 after 72 hrs might be due to proton symport (Tangeny *at al.* 1992). Addition of benezoid amino acids like tyrosine and phenylalanine could increase the enzyme production in a medium devoid of protein source. Among the metal ions, K^+ and Ca^{2+} showed the enhancing effects, but As^{3+}, Pb^{3+} and Li^{3+} did not affect the growth. Addition of antibiotics (100 µg/ml) after one hour of cultivation showed mixed response in the growth and enzyme production. Aminoglycoside antibiotics (Kanamycin and Streptomycin), aromatic antibiotic (Chloramphenicol) and β-lactam antibiotics (Ampicillin) showed complete bactericidal effect, whereas the strain showed resistance against marcolide antibiotic like Erythromycin and replication inhibiting antibiotic like Novobiocin. The effects of the antibiotics were confirmed by filter paper disc diffusion test. However enzyme synthesis was not much affected. This indicated that the secretion of amylase in this species involved liberation of or activation of preformed enzymes rather than *denovo* synthesis, a result contrary to that of β-amylase synthesis by *Bacillus megaterium* B_6 (Ray *et al.* 1996). The natural agent with microbicidal activities, extracted from various plant parts could neither affect the growth nor the enzyme synthesis. Starch acted as the best inducer for enzyme production, followed by dextrin. The optimum concentration was found to be at 0.5%(w/v). Among the waste starches tested, pulse powder showed the best result followed by wheat flour and tapioca (Table. 1). Inhibition of growth and enzyme production in presence of glucose and maltose indicated the inability of the strain to utilize these sugars as sole carbon source and production of some oligosaccharide as the end product of the amylolysis. The amylase was found to have a pH and temperature optima at 7.0 and 60°C respectively, an observation different from that of other *P. stutzeri* amylases (Kainuma, 1988).

Although reports are available on maltotetraose forming *Pseudomonas stutzeri* (Schmidt & John, 1979; Mezaki *et al,* 2001), fermentation of waste starches and the potentiality to be used in waste utilization probably left unchecked. However the observations indicated that the present strain of *P. stutzeri* could be used for commercial production of amylase utilizing starchy wastes. Further, the resistance against antibiotics, toxic metal ions and natural microbicidal agents added some extra

potentiality for using the strain in utilization of starchy effluents released from texile and other industries.

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