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SCREENING OF AN ACTINOMYCETE ISOLATE OF SAMBHAR SALT LAKE, INDIA FOR PRODUCTION OF INDUSTRIAL ENZYMES

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ABSTRACT

Actinomycetes constitute a diverse group of Gram positive mycelial bacteria found in terrestrial, freshwater and marine environments. They are well known producers of bioactive compounds such as antibiotics and are now being studied for the production of industrial enzymes. The majority of currently used industrial enzymes are hydrolytic in action and are used for the degradation of various natural substances. Detergent, textile, pulp and paper industries, organic synthesis and biofuels industry are the major consumers of hydrolytic enzymes. The demand for more stable enzymes in many industrial applications is growing rapidly today, which can be satisfied if actinomycetes from diverse and extreme environments are isolated and studied for enzyme production. In the present study, an actinomycete isolate of Sambhar salt lake, India was characterized and studied for the production of hydrolytic enzymes. The isolate produced three important enzymes protease, amylase and cellulase but it was not able to produce xylanase.

Key words: Hydrolytic enzymes, Extreme environment, Carboxymethyl cellulose, Proteolytic enzymes, Amylolytic enzymes.

INTRODUCTION

In nature, a wide variety of microorganisms exist that secretes extracellular enzymes that can degrade many polymer compounds. Actinomycetes constitute a potential source of biotechnologically interesting substances such as enzymes, antibiotics, enzyme inhibitors and immunomodulators[1]. From traditional times, they are well known as antibiotic producers and now a days being explored on large for the production of industrial enzymes also.

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Enzymes are biocatalysts that perform variety of chemical reactions and are commercially used in many industries such as food, detergent, pharmaceuticals, diagnostics and fine chemicals. Almost 75% of all industrial enzymes are hydrolytic in nature of which carbohydrases, proteases and lipases dominate the enzyme market, accounting for more than 70% of all enzyme sales. It is estimated that this technical enzyme market will increase at a 6.6% compound annual growth rate (CAGR) to reach $1.5 billion in 2015.[2]

Proteases are one of the most important industrial enzymes accounting for nearly 65% of the total worldwide enzyme sales[3]. They catalyze hydrolysis of peptide bonds of proteins[4] by addition of water across peptide bonds. Amylase are low molecular weight enzymes (45000 to 50000D) that catalyzes the hydrolysis of internal α-1, 4 glycosidic bonds in polysaccharides with the retention of α- anomeric configuration in the products and account for about 30% of the world's enzyme production[5,6]. Both these enzymes are widely used in household detergents and in the food processing, animal nutrition, pulp and paper, textile, leather processing, and chemical industries[7,8]. Cellulase are the enzymes that hydrolyze cellulose, the biopolymer abundantly found on the earth surface and account for approximately 20% of the world's enzyme market[9]. After cellulose, xylan is the second most abundant biopolymer and the major hemicellulosic polysaccharide present in the plant cell wall. Enzymatic hydrolysis of it requires xylanase (endo-1, 4-β-D-xylan xylanohydrolase) that degrade the xylan backbone made up of β-1,4-D-xylanopyranosyl residues into small oligomers[10]. These enzymes have received worldwide attention due to their potential applications, especially in the biodegradation of agronomic wastes, and are being increasingly used in textile, paper-pulp and food industries. The new development in biotechnology has extended the spectrum of these enzymes beyond the traditional industries into many new fields such as clinical, medicinal and analytical chemistry. Specific properties of enzymes isolated from extreme environments are expected to result in novel process applications[11,12]. This study was carried out to characterize an actinomycete isolate of Sambhar salt lake, India for the production of industrially important enzymes.

EXPERIMENTAL

Actinomycete isolate

Water samples from salt evaporation ponds of Sambhar salt lake, Rajasthan, India (26°55'12"N and 75°12'00"E) were collected in autoclaved bottles. Actinomycete was isolated on Actinomycetes isolation agar medium (AIA) and maintained on the same at 4° C. The isolate was named as SSL 1.

Characterization of actinomycete isolate

Isolate SSL1 was characterized morphologically and physiologically as per the International *Streptomyces* Project (ISP)[13]. The microscopic characters were studied by cover slip culture method after 4 days of incubation on AIA medium. The presence of aerial and substrate mycelium, their fragmentation, spore chain morphology and pigment production were observed. Colour of aerial and substrate mycelium along with pigment production was also observed on different ISP media, ISP 2, ISP 3, ISP 4, ISP 5, and ISP 6. The observed structures were compared with Bergey's Manual of determinative bacteriology[14]. Various tests as gelatine liquefaction test, H_2S production test, indole production test, methyl red test, Voges Proskauer test, citrate utilization test, catalase test, carbohydrate fermentation, tyrosine utilization test, xanthine utilization test and hypoxanthine utilization test were also performed to characterize isolate biochemically.

Screening of the isolate for the production of industrially important enzymes

Preliminary screening for production of different enzymes was carried out using agar plate assay in which the SSL1 isolate was inoculated on respective media and incubated for 4-6 days at 30^0C. For protease production, SMP agar medium was used[15]. Starch agar medium was used for screening for amylase production. After incubation, the plates were flooded with iodine reagent[16]. Carboxymethyl cellulose (CMC) agar medium was used for secretion of cellulose after which the plates were flooded with an aqueous solution of congo red (1% w/v) for 15 min and then destained with 1.0 M NaCl for 15 min[17]. Xylan agar medium was used for xylanase screening. After incubation, the plates were stained with congo red solution (0.1 % congo red and 5% ethanol in distilled water) for 15 min and destained with 1M NaCl[18]. The width of clear zone of hydrolysis around isolate was measured after each screening assay as a measure of hydrolytic activity of isolate.

Production medium and cultivation conditions

Shake flasks experiments were carried out for only those enzymes for which preliminary screening test were found to be positive. All the experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL M1 broth[19], starch beef broth[20] and carboxymethyl cellulose broth[21] for protease, amylase and cellulase production, respectively. Sterile medium was inoculated with 1% of 24 hrs grown cultures and incubated at 30°C under agitation at 150 rpm for 6 days. Culture was withdrawn at regular time intervals of 24 hrs and centrifuged at 4000 rpm at 4°C for 10 minutes and the cell free supernatant was assayed for protease activity. Centrifugation for amylase and cellulase was performed at 10,000 rpm.

Enzyme activity assay

Proteolytic activity of the supernatant was determined by using spectrophotometeic method, given by Cupp- Enyard[22] with some minor modifications. Casein was used as substrate and tyrosine as standard. One unit of enzyme is defined as the quantity of enzyme required to release 1 micromoles of tyrosine per min, at 37°C and pH 7.5 [23]. α-Amylase activity of the supernatant was determined by iodometric method[24] using starch as standard. One unit is defined as the amount of enzyme causing the removal of 1 mg soluble starch per min at 50°C and pH 7.0. Cellulase activity (CMCases) of the supernatant was determined by using the DNS method[25] using 2% carboxymethyl cellulose as substrate and glucose as standard. One international unit is defined as amount of enzyme causing release of 1 µmol of glucose per min at 50°C and pH 7.0.

RESULTS AND DISCUSSION

Morphological and physiological characterization

The isolate had filamentous nature and mycelium was made up of filaments as seen on direct microscopic observation of inoculated plate under 100X oil immersion. Filament fragments as the mycelium ages. The characters of the colony on different media are summarized in Table 1. Colonies were dry, compact chalky in appearance and revealed Gram positive and acid fast negative nature. Spore chain was found to be of flexible nature. The isolate did not produce any kind of diffusible pigment on different ISP media tested. The biochemical characters of the isolate are shown in Table 2. The filamentous morphology along with the presence of aerial and substrate mycelium of Gram positive nature confirms the isolate to be member of actinomycetes. However, identification by 16S rDNA sequencing is to be carried out.

Table 1: Morphological features of SSL 1

Medium	Incubation time (Days)	Growth	Aerial mycelium	Substrate mycelium
AIA	5	Good growth	White	Yellow
ISP 2	24	No growth	-	-
ISP 3	15	Good	Brown	Brown
ISP 4	18	Good	Grey	Cream
ISP 5	15	Scanty growth	White	White
ISP 6	24	No growth	-	-

Table 2: Biochemical characteristics of SSL1

Biochemical test	Observation
Gelatin liquefication	-ve
H_2S production	-ve
Indole test	-ve
Methyl red test	+ve
Voges Proskauer test	-ve
Citrate utilization test	+ve
Catalsae test	+ve
Carbohydrate fermentation test	
(a) Dextrose	+ve
(b) Sucrose	+ve
(c) Lactose	-ve
Tyrosine degradation	+ve
Xanthine degradation	+ve
Hypoxanthine degradation	+ve

Screening of the isolate for the production of industrially important enzymes

Actinomycete isolate (SSL1), when screened for the production of different enzymes by agar plate assay, was found to produce amylase, protease and cellulase enzymes. A clear zone of hydrolysis around the SSL1 steak was observed. Ratio of width of zone of hydrolysis to the isolate steak width was measured for all enzymes (Table 3) in order to assess the enzymatic activity of the isolate. The isolate was not found to produce xylanase as observed by absence of zone of hydrolysis on xylan agar plate.

Table 3: Enzymatic activity of SSL1

Enzyme	Width of isolate streak (cm)	Width of zone of hydrolysis around streak (cm)	Ratio (zone of hydrolysis/isolate width.)
Protease	0.5	2.0	4
Amylase	0.9	1.2	1.3
Cellulase	0.2	0.5	2.5
Xylanase	0.4	No zone of hydrolysis	-

Enzyme activity assay

Enzymatic activity in the culture supernatants was measured after interval of 24 hours till 144 hours. Protease activity after 24 hrs of growth was found to be 0.12 U/mL and maximum activity was found in the supernatant of 120 hrs old culture (0.32 U/mL) (Fig. 1). α-Amylase activity after 24 hrs of growth was found to be 0.34 U/mL and maximum activity was found in the supernatant of 96 hrs old culture (0.61 U/mL). Thereafter, there was reduction in the activity up to 144 hrs (Fig. 2).

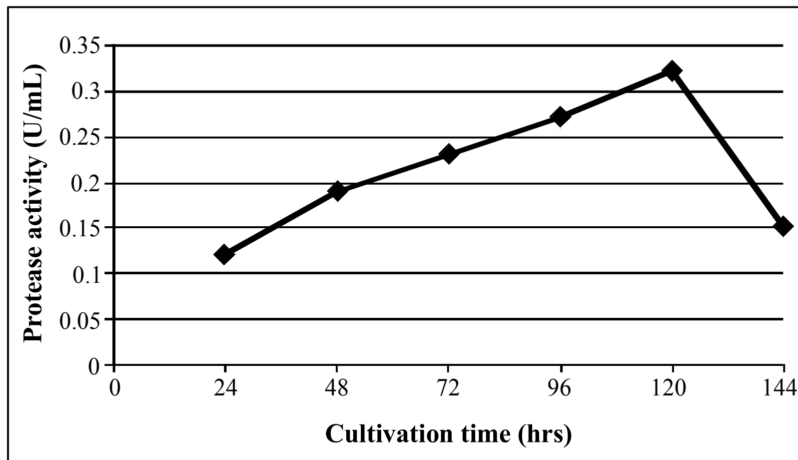

Fig. 1: Protease activity of isolate

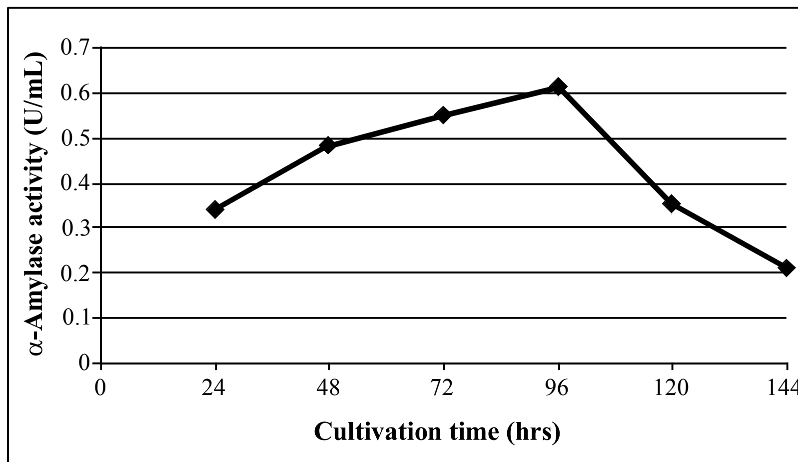

Fig. 2: α-Amylase activity of isolate

In contrast to the results obtained in the present study, α-amylase activity in culture supernatants of an actinomycete strain was reported to be highest after 72 h of incubation at 55°C[26]. Maximum amylase and protease production of *Streptomyces remosus* was also found after 48 and 166 hrs of incubation, respectively at 28°C[27]. Carboxymethyl cellulase activity after 24 hrs of growth was found to be 0.072 U/mL and maximum activity was found in the supernatant of 96 hrs old culture (0.135 U/mL). Thereafter, there was reduction in the activity up to 144 hrs (Fig. 3).

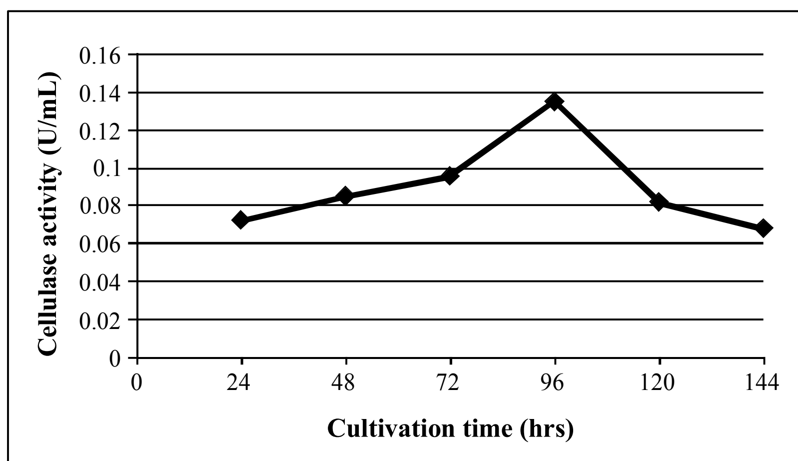

Fig. 3: Carboxymethyl cellulase activity of isolate

In contrast to it, the highest CMCases activity of a *Streptomyces* strain J2 was reported after 72 hrs of incubation at 7 pH and 60°C[9]. Since the isolate SSL 1 was isolated from Sambhar salt lake, a halophilic environment and hence, enzymes produced by this isolate can be used for novel processes. Although the activity of all enzymes produced was not high but the production parameters can be optimized for obtaining higher enzyme activity. These enzymes can also be checked for their stability at higher temperatures and alkaline pH so that they can be used for those industrial processes that require harsh conditions.

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