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ISOLATION AND PRELIMINARY OPTIMIZATION OF *SACCHAROMONOSPORA AZUREA* AP-11/18 FOR ALKALINE THERMOSTABLE LIPASE PRODUCTION

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ABSTRACT:

An actinomycete strain designated as AP-11/18 isolated from edible oil soaked soils of an oil industry unit at Kadi (Gujarat) was identified as *Saccharomonospora azurea* AP-11/18 (MTCC accession no. 10252 and showed 99.443% similarity of 16s rDNA sequencing with NA 128(T). The strain is aerobic, gram positive and with light bluish grey aerial mycelium and dark reverse. Small and round spores with smooth surface are sessile and attached singly on aerial hyphae. The optimum pH is 8.0 and optimum temperature is 40^0C, producing heavy sporulation within 3 days on Bennet's agar. Strain is strongly lipolytic, producing large clearance zones on tributyrin agar plates. Lipase production was optimized in liquid Bennet's medium under shake culture. The strain concurrently produces amylase & protease as well.

Lipase Production parameters like temperature, pH and nutrition e.g. carbon (Lipids and Carbohydrates), nitrogen (organic as well as inorganic) and surfactant were optimized. Optimum enzyme production was obtained at 40^0C at pH 8.0 and 140 rpm.

KEY WORD: *Saccharomonospora azurea,* alkaline thermostable lipase and production.

INTRODUCTION:

Thermophilic Monosporic Actinomycetes with single spores on aerial hyphae are included in the genera ***Thermoactinomyces***, ***Thermomonospora*** and ***Saccharomonospora***. While ***Thermoactinomyces*** has the presence of true bacterial endospores, ***Saccharomonospora*** shows the presence of arabinose and galactose in cell hydrolysates (McCarthy and Cross,1984).

Microbial Lipases, the triacylglycerol acyl hydrolases (E.C. 3.1.1.3), are amongst important biocatalysts with immense applications, carrying out novel reactions in both aqueous and nonaqueous media, utilizing wide spectrum of substrates, being high stable at extremes of temperature, pH and organic solvents, and due to chemo-, regio- and enantioselectivity. The easy

cultivability of microbes provides the microbial lipases an opportunity for industrial uses in a variety of biotechnological fields such as food and dairy (cheese ripening, flavor development), detergents, pharmaceuticals, agrochemicals, oleochemicals and biosurfactants. Lipases can be further exploited in many newer areas where as potential biocatalysts (Saxena., 1999). Extracellular lipases have proved efficient and selective biocatalysts in biosensors, chemicals, leather, and cosmetics thus finding the top most consumption in the biotechnology field, estimated at 2.5 billion US dollars in 2010.

Lipases are produced by microorganisms either singly or together with other hydrolases like esterases. Lipases from fungi and actinomycetes are mostly extracellular enzymes easily fermented, recovered and processed. The biosynthesis of microbial lipases has been shown to be influenced by a variety of parameters including incubation time, temperature, pH, carbon source, nitrogen source as well as surfactants etc.(Kader *et al*., 2007; Sekho *et al.,*2006;Pera *et al.,* 2006).

MATERIALS AND METHODS:

Isolation of Actinomycetes from soil:

Soil samples from 20 different oil industry units at Kadi (Gujarat) were enriched with $CaCO_3$ for 5 to 6 days. The Actinomycetes from these were isolated on Bennet's agar incubated at 40^0C for 7 days, avoiding Bacterial contaminants by the incorporation of Streptomycin and avoiding Fungal contaminants by the incorporation of Nystatin respectively. Morphologically different types of colonies were picked up separately.

Culture Maintenance:

Actinomycete isolates were maintained on Bennet's agar slants (Casein Enzyme hydrolysate: 10gm, Meat extract: 5gm, Yeast extract: 5gm, Glucose: 10gm, KH_2PO_4: 5 gm and Agar: 2.5gm per L. The pH of the medium was adjusted to 8.0 and culture was incubated at 35^0C for 7 days) and stored thereafter in refrigerator at ~ 4^0C. Sub culturing was carried out once in every 2-3 months.

Screening of actinomycetes for extracellular lipases:

Comparison of Lipase production of 21 different isolates on Tributyrin Agar medium (Tri butyrin: 1.0 gm%, Peptone: 0.5gm%, Yeat extract: 0.5gm%, Agar: 2.5gm% was done by measuring diameter of lipid clearance zones. Inoculation was done by 6 mm dia. discs cut from 96 hrs. sporulating growth in the TBA agar plates . The cultures were incubated at 40^0C for 96 hrs. and zone of lipid hydrolysis was measured. 6 superior most strains were selected for further studies in Bennet's broth medium.

Inoculum preparation and Production medium:

50 ml of sterilized Bennet's broth in 250ml Erlenmeyer flasks was inoculated with 6 mm disc culture and incubated at orbital shaker at 140 rpm and 40^{0}C for 96 hrs. when the optimum growth (O.D 2.0 at 600 nm) was achieved. 1.0% of this growth was used for inoculating 50 ml lipase production Bennet's broth medium in 250 ml flasks, keeping composition and growth conditions the same as in growth phase. Lipase in broth filtrate was estimated by colorimetric assay using p-nitro phenyl palmitate (pNPP) by the method reported by Savitha *et al.*, (2007). One lipase unit (U) is equal to the amount of enzyme that liberated 1 µ mole p-nitro phenol per 60 min. 2 best lipase producers in broth medium were selected for further studies on production of Amylase and Protease on TBA medium containing starch /milk but no Tributyrin. Effect of agitation on lipase yields for the 2 strains was compared under static/ agitated fermentation on rotary shaker as above. Optimization of parameters for Lipase production by highest lipase producing strain (AP 11/18) was carried out.

Cultural characteristics and identification of strain AP 11/18 :

The highest lipase producing strain (AP 11/18) thus obtained was cultured on Bennet's agar, Calcium malate agar, Czapex sucrose agar, Glucose aspargine agar, Glycerol aspargine agar, Nutrient agar and Potato dextrose agar as suggested by Xiang *et al.*, 1998; Runmao., 1987 & Runmao *et al.*,1988. The strain was subjected to biochemical characterization by testing the utilization of sugars / substrates and production of enzymes as per Runmao *et al.*, 1988 & Sheikha *et al.*, 2002.

Optimization of Medium Parameters for lipase production:

The optimization of fermentation parameters: temperature (25^{0}C, 30^{0}C, 35^{0}C, 40^{0}C, 45^{0}C, 50^{0}C, 55^{0}C & 60^{0}C) , pH (7.0, 8.0, 9.0, 10.0 and 11.0), Carbon sources, Nitrogen sources and Surfactant for lipase production was done by taking one particular parameter at a time, using varying values. (Kader *et al.*, 2007; Gulati *et al.,* 2005; Simkhada *et al.*, 2007; Selva *et al.*, 2008; Jagtp *et al* ., 2010.)

RESULTS AND DISCUSSION:

Isolation of Actinomycetes for the production of extracellular lipase:

Six strains out of the 21 actinomycete isolates (AP11/1, AP11/2, AP11/5, AP11/6, AP11/18 & AP11/21) exhibited zone of lipid hydrolysis on TBA agar plates at 96 hrs. incubation greater than 15 mm. AP11/18 showed highest lipase activity (172U/ml) when fermented in Bennet's Broth medium. The next highest was AP11/6 yielding only 75 U/ml (Fig.1).

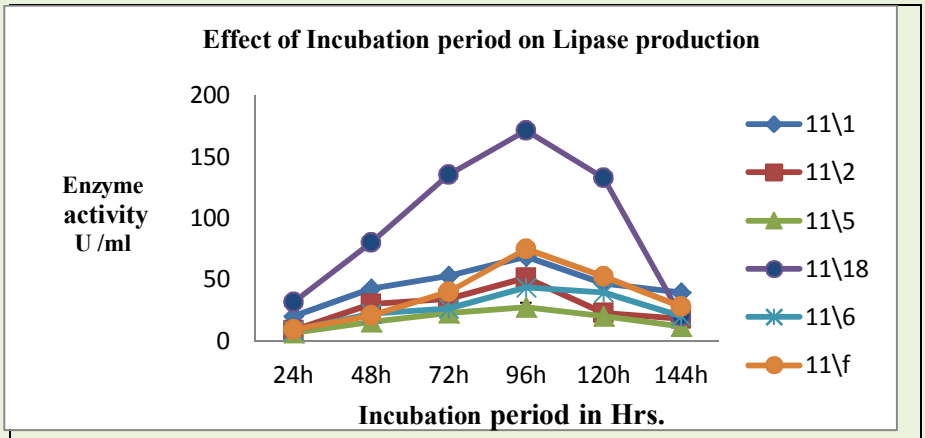
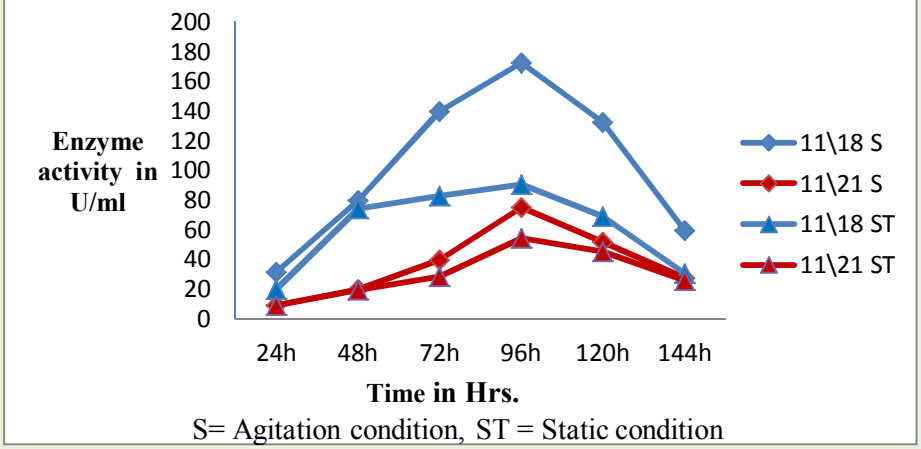


Fig.1. Lipase production pattern of 6 Actinomycete strains from Oil mill compound soils

These 2 cultures were taken up for study of the effect of agitation on the lipase yields in Bennet's Broth medium. Fig. 2 depicts these results.



S= Agitation condition, ST = Static condition

Fig.2. Effect of Agitation and Static condition on Lipase production

Agitation resulted in increased lipase titers, indicating aerobic pathway for lipase biosynthesis.

Cultural characteristics:

Actinomycete AP-11/18 was found to be gram positive with light bluish grey aerial mycelium and dark reverse on Bennet's Agar medium. Microscopic observations confirmed sessile, small & round spores with smooth surface attached singly on the conidiophores (monosporic).

Table 1. Presents morphological characteristics of Actinomycete AP 11/18 on various media. The sparse aerial mycelium on most media was white till greenish grey sporulation covered the growth. This strain did not produce any diffusible pigment on all media tested. Observation of very small colony on all media. Bennet's agar as well as on Nutrient Agar slightly raised growth observed while remaining all gives flat growth.

Table 1: Cultural characteristics of Actinomycete AP-11/18.

Medium	Growth	Mycelium colour	Reverse	Soluble pigment
Bennet's agar	+++	Dark greenish grey	Dark blackish	Black
Czapex sucrose agar	+	Light White	Colourless	None
Glucose aspargine agar	+++	Dark white	Light pale Yellowish	None
Glycerol aspargine agar	++	White gray	Greyish white	None
Nutrient agar	+	Whitish gray	Yellowish gray	None
Potato dextrose agar	+++	White	Pale Yellowish	None
Calcium malate agar	++	White	colourless	None

Table 2. shows the data on biochemical characterization of Actinomycete strain AP 11/18. The carbohydrates Sucrose, Glucose, Trehalose, Raffinose, Dextrose, Galactose , Melibiose, Mannose and Fructose gave good growth. No growth observed with lactose, xylose, maltose, Arabinose , Manitol. Phospatase, Urease, Catalase, Amylase, Protease, Lipase and β galacosidase production were observed by the culture. Citrate utilization, Nitrate reduction, ONPG utilization Arginine utilization were positive while Malonate utilization and acetoin production were negative. Strain is resistant against Antibiotic Niastatine and Streptomycine at the concentration of 50 µg/ml.

Table 2: Physiological and biochemical characterization of Actinomycete AP-11/18.

Character	Utilization	Character	Utilization
Carbon source utilization:		Enzyme production:	
Arabinose	_	Catalase	+
Dextrose	+	β-galactosidase	+
D-fructose	+	Urease	+
Galactose	+	Amylase	+
Glucose	+	Protease	+
Maltose	_	Alkaline Phosphatase	+
Mannose	+	Nitrate reduction	Weak +
Mannitol	_	Antibiotic resistance:	
Melibiose	+	Niastatine (50µg/ml)	+
Raffinose	+	Streptomycine (50 µg/ml)	+
Sucrose	+	Citrate utilization	+
Trehalose	+	Arginige utilization	+
Xylose	_		

Strain:

In absence of the electron microscope photography of the sporulating mycelium, the culture appeared to be a monosporic actinomycetes based on the above characters. The strain was

deposited for identification by 16s rDNA technique to MTCC and was confirmed as *Saccharomonospora azurea* AP-11/18 (MTCC accession no. 10252 and showed 99.443% similarity of 16s rDNA sequencing with NA 128 (T).

Effect of temperature and pH on lipase production:

The lipase production at 96 hrs. was found to be highly temperature sensitive with an optimum at 40ºC, even a slight change in temperature resulted in decline of yields. Particularly at temperatures above 50 ºC there was cessation of lipase production (Fig.3a). Slightly alkaline pH (7.5 to 9.0) was observed to be the optimum for lipase yields, though the change in pH did not exert very significant impact indicating alkaliphilic nature of the organism and the enzyme (Fig.3b).

Fig: 3a Effect of Temperature on lipase production by *Saccharomonospora azurea* AP 11/18

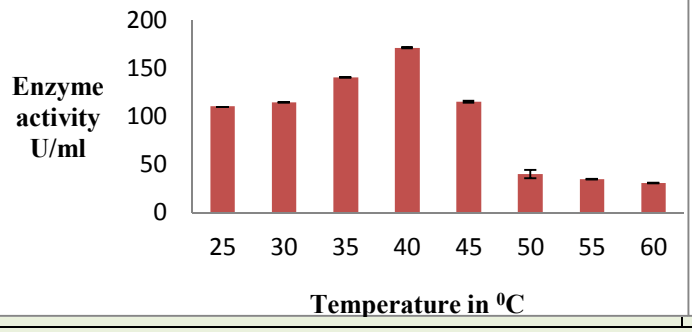
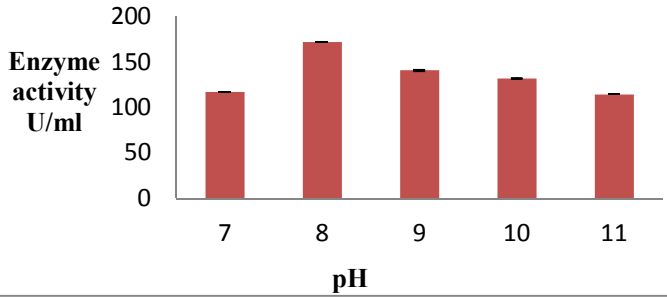


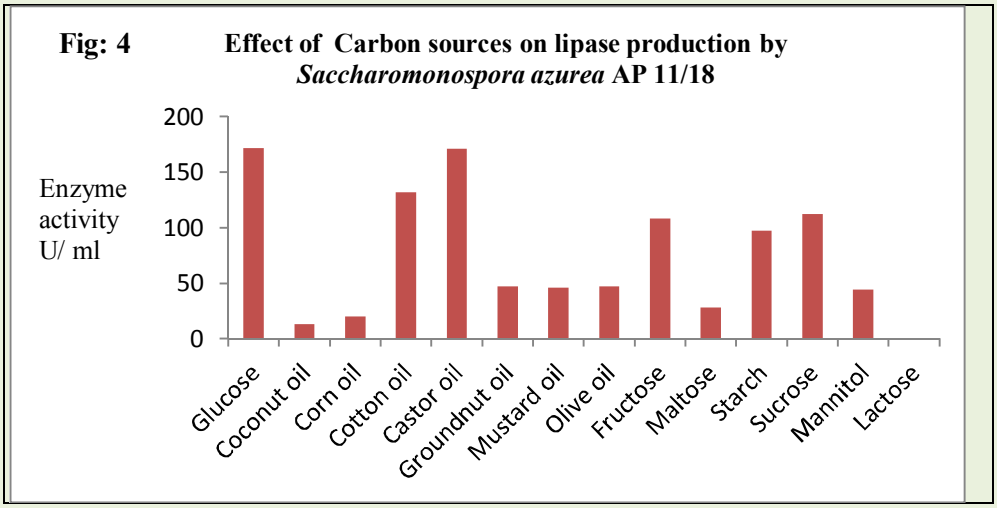
Fig: 3b Effect of pH on lipase production by *Saccharomonospora azurea* AP 11/18



Effect of Carbon source on lipase production:

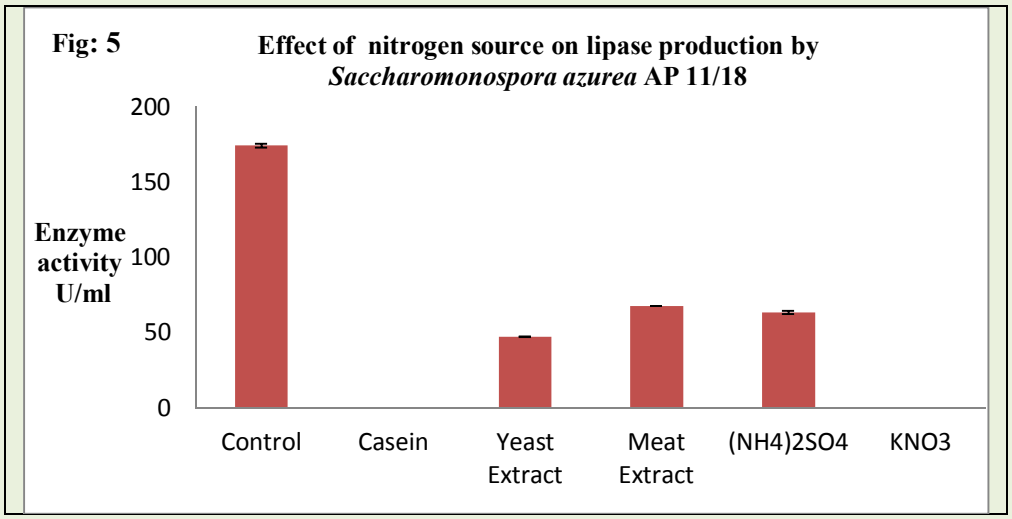
Substitution of 1% Glucose (from basic medium – Bennet's broth) by 1 % carbohydrates: fructose, maltose, starch, sucrose, mannitol & lactose or 1 % vegetable oils: Castor oil, coconut oil, corn oil, cotton seed oil, ground nut oil, mustard oil, and olive oil as C source influenced the lipase production significantly with Glucose and Castor oil proving to be superior most standalone C source for lipase production by *Saccharomonospora azurea* at 96 hrs. (Fig. 4). Lactose resulted in no lipase production.

Fig: 4 Effect of Carbon sources on lipase production by *Saccharomonospora azurea* AP 11/18



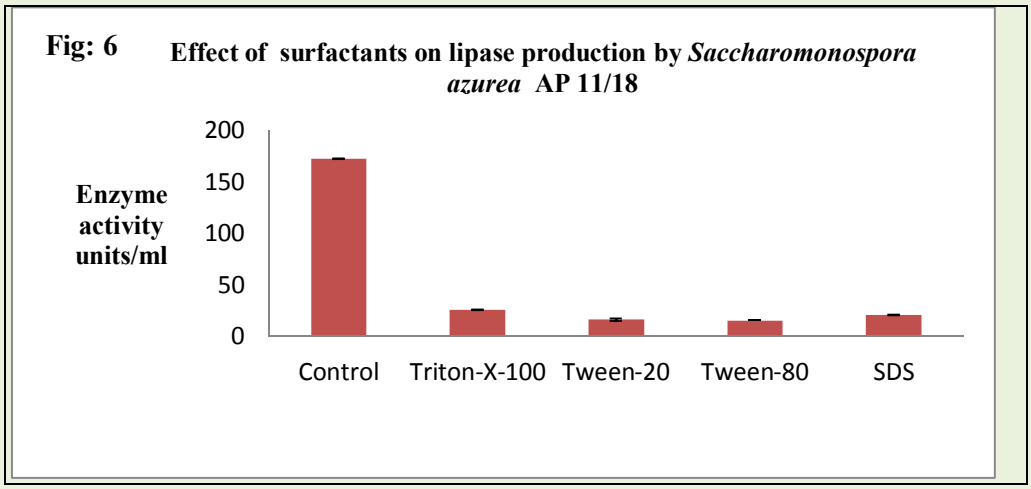
Effect of nitrogen source on lipase production:

Substitution of a total of 2.0% organic nitrogen source (from Bennet's broth) by 0.5 % standalone Casein enzyme hydrolysate , Yeast extract, Meat extract, $(NH_4)_2SO_4$ & KNO_3 was provided for lipase production by *Saccharomonospora azurea* at 96 hrs. (Fig-5). Control containing combination of Casein enzyme hydrolysate , Yeast extract and Meat extract as N source. The original nitrogen source of Bennet's Broth gave best yields rather than standalone nitrogen sources. In media containing standalone Casein enzyme hydrolysate and KNO_3 lipase production was not detected.



Effect of Surfactant on lipase production:

The presence of surfactants (ionic as well as non-ionic), including Triton X-100, Tween 20, Tween 80 and Sodium Dodecyl Sulphate (SDS), inhibited the Lipase activity of strain AP11/18 drastically at 0.45% concentration. A significant observation was that the growth of the organism was also drastically low when these surfactants were incorporated.



CONCLUSIONS:

The actinomycetes isolated from soil samples of Oil mill compounds were screened for lipase production. Actinomycete AP-11/18 was the best producer on Bennet's agar / broth media. The enzyme was found to be inducible, extracellular, alkalophilic lipase with optimal activity 96 hrs obtained at a pH of 8.0 and 40ºC incubation under rotary shaking. This is first report of such high yields in short fermentation cycle by *Saccharomonospora azurea.* Glucose and Castor oil proved to be best C sources and organic peptone nitrogen as best N source. Surfactants depressed the enzyme production. Further work is desirable for optimizing a commercializable protocol for production of this enzyme for exploring its applications.

The culture was subjected to biochemical characterization and the genetic identification through 16s rDNA analysis by Microbial Type Culture collection confirmed it as *Saccharomonospora azurea*.

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