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Solid-state Lipase Production by *Staphylococcus cohnii* AP-CMST using Anchovy Processing Wastes

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A bacterium *Staphylococcus cohnii* AP-CMST, capable of producing lipase was isolated from the gut of marine fish *Sardinella longiceps*. The lipase production was investigated in solid-state fermentation experiment using anchovy processing wastes. The time course for lipase production inferred that 72 h was the optimum duration for higher lipase production. Effect of carbon and nitrogen sources supplementation on lipase production revealed that sorbitol and beef extract aided the higher lipase production than the other tested carbon and nitrogen sources. The suitable surfactant and triglyceride observed to increase the lipase production were poly ethylene glycol and palm oil respectively. Effect of trace elements on lipase production showed that only calcium chloride and magnesium sulphate have the inducing effect on lipase production. The halotolerancy of *S. cohnii* AP-CMST for lipase production indicated that 4% of sodium chloride was optimum to yield maximum lipase. The effect of physical parameters on lipase production revealed that pH 8 and 40°C temperature were optimum for higher lipase production.

Key words: Anchovy processing waste, Lipase, NaCl palm oil, *Staphylococcus cohnii.*

Lipases (EC 3.1.1.3) are glycerol ester hydrolases that catalyze the hydrolysis of triacylglycerols into fatty acids, di-acylglycerols, mono-acylglycerols and glycerol fatty acid, in oil-water interface[1]. While lipases are widely distributed in animals, plants, bacteria, yeast, and fungi, nevertheless, microbial lipases are commercially significant. The lipase producing fungi includes the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp. The important lipase producing yeast genera belonging to *Candida* sp, *Yarrowia* sp., *Rhodotorula* sp., *Pichia* sp., *Saccharomycopsis* sp. and *Torulaspora* sp. Among microbial lipases, bacterial lipases are very important, because they exhibit some good properties that make them potential candidates for biotechnological industries. They include the genera of *Bacillus* sp., *Pseudomonas* sp., *Burkholderia* sp., *Serratia* sp., *Acinetobacter* sp. and *Staphylococcus* sp.[2].

Among the lipase producing bacterial populations *Staphylococcus* genera is very important one. Lipases with wide substrate specificity and different biochemical properties have been identified and characterized from *Staphylococcal* lipases. They have wide substrate specificity with different biochemical properties.

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The important lipase producing *Staphylococcal* species includes *S. warneri*, *S. haemolyticus*, *S. saprophyticus*, *S. caseolyticus, S. xylosus, S. epidermidis*[3-8]] etc.

Solid-state fermentation (SSF) of waste substrates for enzyme production is a possible technique that reduced the production cost. Among the waste substrates, fish processing wastes are generally rich in protein, due to its good amino acid balance and protein content that can be used as a good solid medium for SSF. Despite this, studies related to the exploitation of such marine waste substrates and by products from fish processing industries for the production of metabolites is very much wanted. In earlier studies, *Sardinella* meal was used as a good substrate for protease and lipase production by bacteria (*Bacillus subtilis* and *Pseudomonas aeruginosa* MNF) and fungi (*Rhizopus oryzae*) respectively[9-11]. Also Esakkiraj *et al.* used tuna fish waste for lipase production by *S. epidermidis* CMST Pi 2 [12]. Considering the above said facts, the present study was undertaken to optimize the lipase production by *Staphylococcus cohnii* AP-CMST isolated from the gut of marine fish *Sardinella logiceps* using anchovy processing wastes.

MATERIALS AND METHODS

Microorganism and Solid-state fermentation

The potent lipase producer *Staphylococcus cohnii* AP-CMST (GenBank accession no. HM582690) previously isolated from marine fish *Sardinella longiceps* was used in this study. For solid-state fermentation (SSF), the bacterium was enriched first using enrichment medium containing beef extract (0.15%), peptone (0.5%) NaCl (3.0%) and glucose (2 %) at pH 7 for 24 h. Then 2% of enriched seed culture was inoculated in 250 ml flask containing 10 ml mineral medium containing 5g powdered anchovy processing waste obtained from local seafood processing industry. The composition of mineral medium was KH_2PO_4 – 0.1%, NaCl-2%; $MgSO_4$ – $7H_2O$ – 0.01% and NH_4NO_3 – 0.5%. The culture was then incubated for 72h and room temperature. All optimization experiments were carried out at 80% moisture level. After 72 h, 50 ml of distilled water was added and placed into a shaker at 150 rpm for 1h. The cells were filtered by using cotton cloth and then centrifuged at 10,000 rpm for 15 minutes and the supernatant was used for further assay.

Lipase assay

Lipase activity was determined spectrophotometrically, using p-nitrophenyl palmitate as the substrate. One unit of lipase activity was defined as the amount of enzyme required to release 1 µmol *p*-nitrophenol released under standard assay condition[13]. The activity was presented as units per gram of dry substrate (U/gds)

Optimization of culture conditions for solid-state lipase production

To find out the optimum time for lipase production, the lipase production was assayed at every 24 h of fermentation up to 144 h and this was carried out by using basal mineral medium.

The different carbon sources (1% w/v) such as glucose, fructose, sucrose, lactose, maltose, mannitol, rhamnose, galactose, strach corn, soluble starch, xylose, and sorbitol were individually tested to the mineral medium. To select suitable nitrogen source (2% w/v) for higher lipase production, the following nitrogen sources like, ammonium nitrate, potassium nitrate, sodium nitrate, ammonium sulphate, ammonium chloride, ammonium hydrogen carbonate, urea, soyabean meal, yeast extract, beef extract, peptone and skim milk powder were individually tested.

Triglycerides (1% w/v) such as castor oil, coconut oil, olive oil, cod liver oil, palm oil, neem oil, gingilly oil, sunflower oil and tributyrin were individually tested for their influence on lipase production. Suitability of various surfactants such as tween 20, tween 40, tween 60, tween 80, triton X 100, PEG, (Poly Ethylene Glycol) and SDS (Sodium Dodecyl Sulphate) were individually tested at the level of 0.2% (w/v). For the selection of suitable metal ions source for lipase production by this bacterium, ten different metal ions were screened (0.02% w/v) as follows zinc chloride, magnesium chloride, barium chloride, mercuric chloride, cobalt chloride, manganeous sulphate, EDTA, zinc sulphate, copper sulphate and calcium chloride.

As the bacterium is a marine isolate, the effect of various concentrations of sodium chloride (2, 4, 6, 8, 10 and 12% w/v) was screened for its efficiency to produce lipase.The effect of temperature on lipase production was studied by

incubating the substrate inoculated with bacterial culture at various temperatures such as 10, 20, 30, 40, 50 and 60°C. Optimum pH for solid-state lipase production was determined by using different pH in the production medium, for which the medium was individually prepared (before autoclaving) with pH 4, 5, 6, 7, 8, 9 and 10.

RESULTS AND DISCUSSION

The effect of incubation time on lipase production showed that 72 h was the optimum duration for maximum lipase production (7.8 U/gds). Above this period the lipase production started to decrease. This is because, the cells may reach the decline phase and displayed low lipase synthesis (Fig 1).

The effect of carbon sources on lipase production revealed that sorbitol has more influence than other sugars (28.5 U/gds). Most of the carbon sources screened have negative impact on lipase production when compared to control (10.9 U/gds) (Fig 2). It was observed that sorbitol is an important agent to improve the lipase activity. Studies by Kambourova *et al.* and Nawani and Kaur proved that sorbitol was the vital agent that improves the stability of lipase produced by *Baciillus stearothermophilus* and *Bacillus* sp., respectively[14, 15]. The present study also finds support from the studies of Sheriff *et al.* and Abdul Rahman *et al.* on the positive effect of sorbitol on lipase production by *Bacillus* sp. and *Pseudomonas* sp [16, 17].

Nitrogen source is an important nutrient factor in fermentation studies and the supply of suitable nitrogen source will favour the production of enzyme or metabolite in higher level. In the present study, the effect of various supplementary nitrogen sources on lipase production under SSF revealed that beef extract (35.9 U/gds) and yeast extract (32.16 U/gds) favored the fermentation positively and they yielded higher lipase production. Among the tested nitrogen sources, ammonium nitrate (0.7 U/gds), ammonium chloride (0.5 U/gds) and potassium nitrate (1.52 U/gds) yielded very low lipase production (Fig 3). Being organic sources beef extract have free amino acids, carbohydrates, essential fatty acids, and it could

Table 1. Effect of initial pH and incubation temperature on lipase production

Initial pH	Lipase activity (U/gds)	Incubation temperature (°C)	Lipase activity (U/gds)
3	13.3	10	22.1
4	18.9	20	25.5
5	22.3	30	34.9
6	28.6	40	46.5
7	31.5	50	41.2
8	40.6	60	30.6
9	36.8	70	25
10	34.7	80	15.0
11	21.1		
12	10.2		

serve as multi growth promoter. This present study results correlates with the earlier studies of Sun and Xu, on the positive influence of beef extract on maximizing the lipase production by *Rhizopus chinensis*[18]. This is also in consistence with the study of Singh *et al.,* where the lipase production by *P. aereoginesa* was enhanced by beef extract[19].

Surfactants are the one of the important factors in fermentation experiments and in particular they induce lipase fermentation, because they act as surface active agent in fermentation and may act as a nutrient source and on lipase production. The present study on the effect of various surfactants on lipase production revealed that Poly ethylene glycol was the suitable inducer for maximum lipase production (24.1 U/gds) (Fig 4). Here the tweens did not support the lipase production. The other surfactants such as SDS, Triton X 100 were also found to enhance the lipase production. This may be due to change of cell wall

permeability or may be the effect of surfactants on cell bound enzyme. The previous report by Alkan *et al.* stated that the surfactants such as SDS, Triton X 100 and PEG supported maximum lipase production by *Bacillus coagulans*[20].

Triglycerides are found as either inducer or inhibitor in lipase production. The present results revealed that palm oil had good effect on lipase production (65.73 U/gds). The coconut oil (6.5 U/gds) acted as an inhibitor for lipase production and exerted negative effect over the control (10.7 U/gds) (Fig. 5). The positive effect of palm oil on lipase production may be due to induction of lipase by oil substrates. This fact has been proved in lipase production by *Bunrkhoderia cepacia*, where the lipase production is highly enhanced by palm oil supplied medium[21]. Moreover, the oil sources also serve as potential carbon and energy sources. There are many reports available for the effect of lipids sources on lipase production. Rajendran and Thangavelu, reported that the lipase production by *Bacillus sphaericus* was induced by seasame oil[22]. Also Abdul Rahman *et al.* suggested that the lipase production by *Pseudomonas* sp. was maximized by olive oil[17].

Trace elements are one of the basic and important components in fermentation experiments, especially in enzyme production. Because enzymes like lipase, protease, amylase etc. require specific metal ions for their activity and stability. The present study on the effect some trace elements such as metal ions on lipase production under SSF revealed that only calcium chloride (19.5 U/gds) and magnesium sulphate

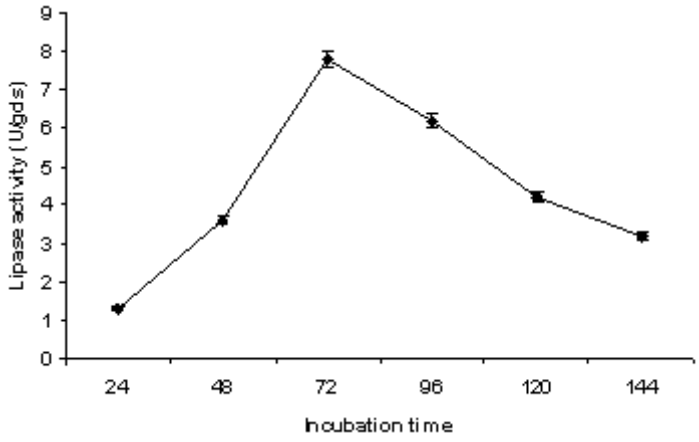

Fig. 1. Effect of incubation time on lipase production

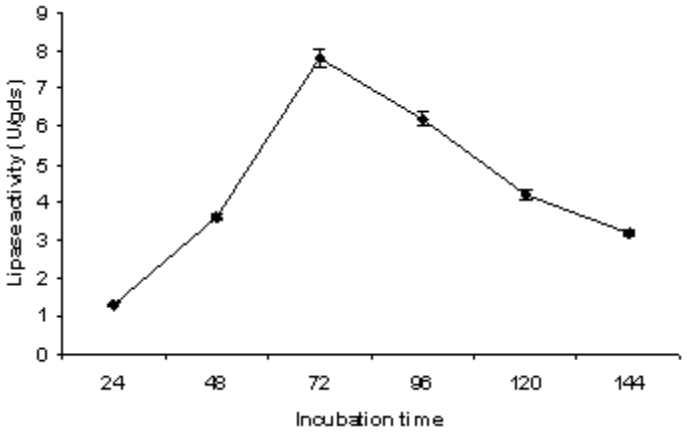

Fig. 2. Effect of carbon sources on lipase production

(13.13 U/gds) gave its maximum influence on lipase production (Fig. 6). These metal ions may helpful to increase the enzyme activity through attachment with the carboxylate side chain groups of aspartyl and glutamyl residues of the enzyme[20]. Similar results were reported on lipase production by *B. coagulans*, where the productivity under SSF of melon wastes was maximized when the medium supplied with $CaCl_2$ [20]. This study also finds significance with the previous studies on lipase production by *B. stearothermophilus,* where the lipase production increased when $CaCl_2$ is added[14].

Halophilic enzymes have very important industrial application and now days the application of halophilic enzymes have come into focus. In the present study, the selected bacterium was halophilic in nature, and hence the effect of various concentrations of NaCl was tested and resulted that 4% (40.6 U/gds) favored maximum enzyme production (Fig 7). So these results showed the bacterium was halophilic and absolutely require NaCl for its growth and enzyme production. But

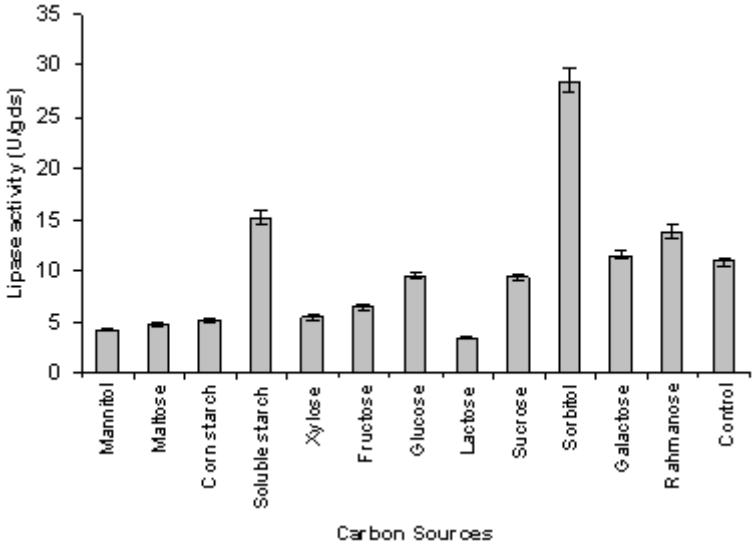

Fig. 3. Effect of Nitrogen sources on lipase production

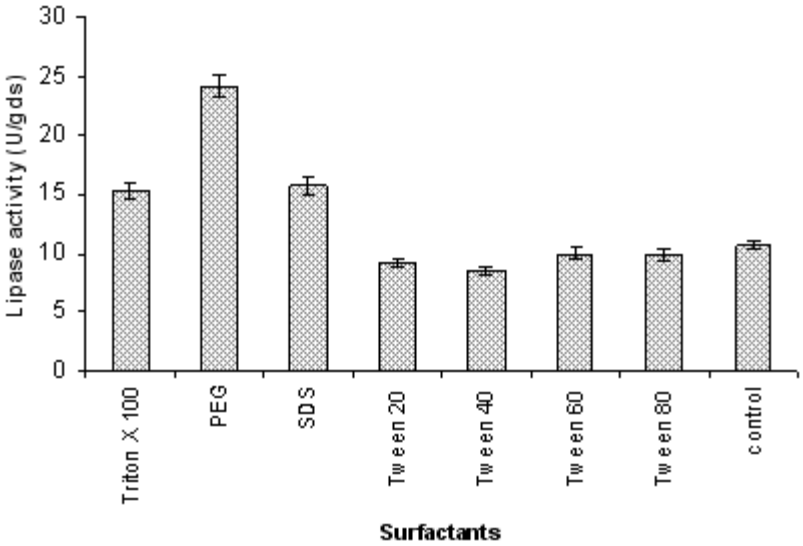

Fig. 4. Effect of surfactants on lipase production

the NaCl concentration beyond the optimum level did not favour the enzyme production and thus it may be due to osmotic problem faced by the host bacterium. Studies related to halophilic enzymes especially lipases are very much limited and only few halotolerent lipase producers were reported. Amoozegar *et al.* reported that *Salinivibrio* sp isolated from saline environment of Iran required 4% NaCl for optimum lipase production[23]. Seghal Kiran *et al.* observed that lipase production by marine *Pseudomonas* sp. require 1.5-2 % NaCl for maximizing the lipase production[24]. The bacterium *Staphylococcus warneri* requires optimum salt concentration of 2.5 M NaCl for maximizing lipase production[25].

The physical parameters are very much effective in any fermentation process for successive production of biomolecules. In the present study, the effect of pH on lipase production revealed that pH 8.0 was the optimum for maximum lipase production (40.6 U/gds) under SSF. Above this level the enzyme production found to be decreased. It showed that the bacterium selected for the present study was alkaline in nature, and also it was observed that, the organism can not survive much at high alkaline condition, therefore the enzyme production was decreased at high pH 12.0 (10.2 U/gds) (Table 1). Because the metabolic activities of microbes are very much sensitive to the changes in pH range, so changes will affect

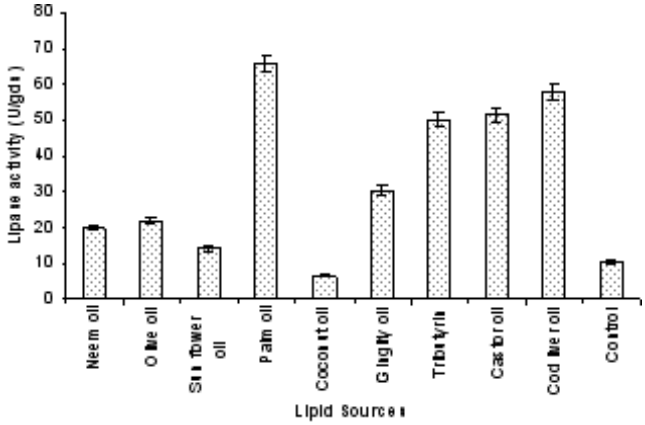

Fig. 5. Effect of lipidic substrates on lipase production

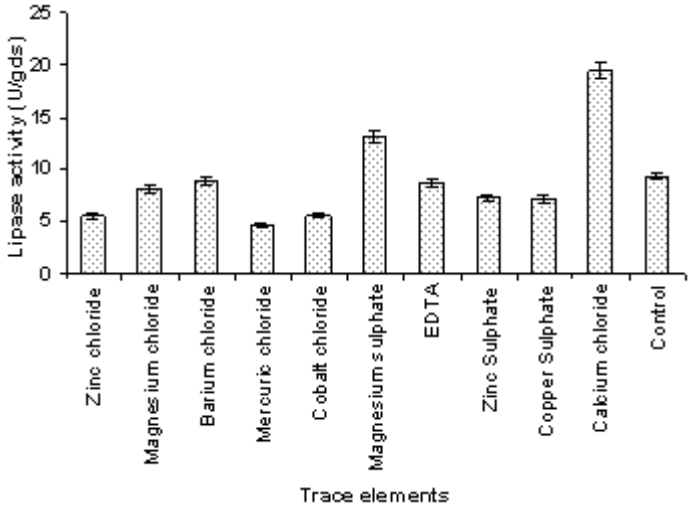

Fig. 6. Effect of trace elements on lipase production

the productivity. This was reported in lipase production by *Corynebacterium paurometabolum* and this bacterium produces maximum amount of lipase at pH 8-8.5, and lipase production decreased when rise in pH above 8.5 [26]. Similar trend was also observed in lipase production by *Pseudomonas aeruginosa,* where it was high at pH 8.0 [19].

In the present study the temperature effect on lipase production inferred that, 40°C was found as an optimum for higher lipase (46.5 U/gds) production. The increase in temperature beyond this optimum level did not favor enzyme production (Table 1). Like pH effect, microbes also responsive to change in temperature, because higher temperature may arrest the metabolic activity of microbes and also favors the heat inactivation. Similar to the present study, Kamzolova *et al.* observed that lipase production by *Yarrowia*

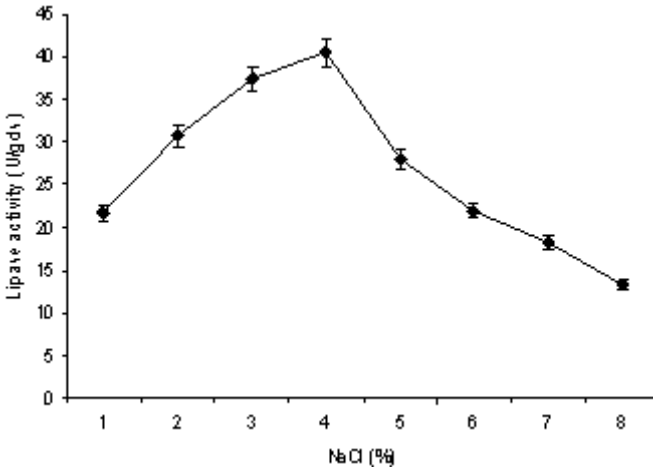

Fig. 7. Effect of different concentration of NaCl on lipase production

lipolytica was higher in fermentation carried out at the temperature of 30°C - 35°C [27]. Destain *et al.* reported that 37°C temperature as the optimum for lipase production by *Yarrowia lipolytica*[28].

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