Solid state production of Esterase using groundnut oil cake by fish intestinal isolate *Bacillus circulans*

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Abstract

A bacterium that produces esterase was isolated from the gut of marine fish *Sardinella longiceps* and identified as *Bacillus circulans.* The esterase production was optimized by using economically cheap agro industrial substrate groundnut oil cake under solid state fermentation. The effect of triglycerides and surfactants on esterase production indicated that the maximum amount of esterase production was observed in castor oil and Tween 80 added medium. The suitable NaCl concentration observed for maximum enzyme production was 4%. The effective nitrogen and carbon sources that enhanced the esterase production were found to be ammonium hydrogen carbonate and maltose. The effect of various kinds of metal ions inferred that, $CaCl_2$ added medium favored the maximum amount of esterase production. The optimum pH, temperature and moisture level for the excellent esterase production were found to be 9, 60^0C and 75%, respectively. The effect of various organic solvents revealed that hexane (102%), paraffin liquid (104%) and nonane (107%) were found to increase the esterase production over the control (100%).

Keywords: Fish gut bacterium, esterase, ground nut oil cake, castor oil.

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Introduction

Esterases and lipases are the enzymes catalyzing the hydrolysis of ester bonds and are widely distributed in animals, plants and microorganisms. In organic media, they catalyze reactions such as esterification, intesterification and transesterification (Kawamoto et al., 1987). Esterases differ from lipases mainly on the basis of substrate specificity and interfacial activation (Long, 1971). Esterases found in plants, animals and microbes, but microbial sources are considered as prominent for esterase production. Because they can be engineered for production of esterase with desirable properties for industrial need. The microbial sources include bacteria, fungi, yeasts and actinomycetes. The bacterial sources reported to produce esterase are *Bacillus licheniformis* (Torres et al., 2005), *Bacillus megaterium* (Jung et al., 2003), *Bacillus* sp (Bakir Ateslier and Metin, 2006), *Sulfolobus tokodaii* (Suzuki et al., 2004), *Streptococcus thermophilus* (Liu et al., 2001) and *Pseudomonas* sp. (Kim et al., 2002). The fungal species include *Trichoderma* sp. (Maeda et al., 2008), *Ophistoma* sp. (Calero-Rueda et al., 2002), *Pencillium* sp. (Horne et al., 2002), *Aureobasidium pullulans* (Kudanga et al., 2007) etc. The yeast include *Saccharomyces* sp. (Lomolino et al., 2003), *Candida* sp. (Ghosh et al., 1991) and *Kluyveromyces marxianus* (Monti et al., 2008). The actinomycetes are known to produce esterase are *Streptomyces coelicolor* (Soror et al., 2007) and *Streptomyces* sp. (Nishimura and Inouye, 2000).

The applications of esterases are found in various fields, they are largely involved in inorganic synthesis process. They are mainly used in pharmaceutical industries to produce chiral drugs such as anti inflammatory drugs to be used as a pain killing agents (Quax and Broekhuizen, 1994). They have application in detoxification of organophosphorous compounds found in insecticides in agricultural industries (Horne et al., 2002). In food application, they are mainly used in dairy industry for flavor enhancement process of dairy products. The other food applications are beverages production (wine and beer), fruit juice processing etc. Esterase is also used in paper, textile, leather and baking industries (Panda and Gowrishankar, 2005).

Production of enzymes in Solid-State Fermentation (SSF) using moist solid wastes or residues from agroindustrial and food industries has plentiful advantages. The main advantage is waste utilization through the production of value added products. Being rich in nutrient content, groundnut oilcake can be used as carbon and energy source for solid substrate for the production of enzymes. Considering the importance of the agro industrial wastes, the present study was undertaken to optimize the culture conditions for esterase production by *Bacillus circulans*

Materials and Methods

1. Bacterium and esterase activity

The bacterium used in this study was isolated from the gut of marine fish *Sardinella longiceps* collected from Colachel coast of Kanyakumari District, Tamilnadu, India. The bacterium produce clear zone after 48 h of incubation when streaked on sprit blue agar supplied with tributyrin. The bacterium was identified as *Bacillus circulans* according to the standard key of Bergey's manual of Determinative Bacteriology and 16s rRNA type sequencing.

2. Solid-state fermentation

Commercially available low cost agricultural residue, groundnut oil cake was used as substrate in this study and it was obtained from a local market. 5g of dry substrate was taken into a 250 ml Erlenmeyer flask and to this a salt solution (10 ml) containing NH_4NO_3-0.20%; K_2HPO_4-0.1% ; $MgSO_4.7H_2O$-0.20% ; NaCl-2% and 0.20% trace metal solution ($MnSO_4$-0.08% ; $ZnSO_4$-0.17% and $FeSO_4$-0.20%) and distilled water was added to adjust the required moisture level. The contents in the flask were mixed thoroughly, autoclaved, cooled to room temperature and inoculated with 24 h old fresh culture of *B. circulans* grown on enrichment broth (beef extract-0.15%, peptone-0.5%, NaCl- 1% and glucose -0.5%; pH 7). Fermentation was carried out at 32 ^{0}C for 48 h with initial moisture level of 70% and inoculum size of 2 ml. All the experiments were done in three sets and average values were reported.

3. Enzyme extraction and Esterase assay

After incubation, 50 ml of distilled water was added into fermented solid and placed into a shaker at 150 rpm for 1 h. Then it was filtered through muslin cloth and was harvested by centrifugation at 10,000 rpm for 15 min. The supernatant was used for further assay. The assay system consists of 1 ml Tris buffer (pH 7.2), 1 ml p-nitrophenyl acetate (100 mM) and 0.5 ml culture supernatant. The mixture was incubated for 15 min at room temperature and then kept in ice box for 5 min. Then the absorbance was read at 405 nm using UV-Vis spectrophotometer (TECOMP 8500). The amount of esterase produced was determined with the help of p-nitrophenol standard graph. One unit of esterase activity is equivalent to one micromoles of p-nitrophenol released under standard assay condition.

4. Media Optimization

4.1 Effect of triglycerides on esterase production

Triglycerides such as castor oil, coconut oil, olive oil, cod liver oil, palm oil, neem oil and gingili oil were tested individually at the concentration of 0.05 ml/g dry substrate into production medium with ground nut oil cake and the medium without any of the above triglycerides was taken as control.

4.2 Selection of surfactants for esterase production

To study the surfactant induced lipase production, five different surfactants were tested (Tween 20, Tween 40, Tween 60, Tween 80 and Triton X 100). The selected surfactants were incorporated individually into the production medium at the concentration of 0.05 ml/g dry substrate and the medium without any of the above surfactant was taken as control.

4.3 Effect of various concentrations of sodium chloride on esterase production

As the bacterium is marine isolate, here the effect of various concentrations of sodium chloride was tested for its efficiency to produce esterase. For this, the production medium was supplied with different concentrations of sodium chloride. The tested concentrations were 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11%.

4.4 Effect of carbon and nitrogen sources on esterase production

To determine the most favorable source of carbon for esterase production, various carbon sources such as glucose, fructose, sucrose, lactose, maltose, sorbitol, mannitol, raffinose, galactose, starch soluble and starch corn were supplemented individually in the production medium at the concentration of 0.1 g/g dry substrate and medium without carbon source was taken as control.

Organic nitrogen sources included beef extract, yeast extract, peptone, skim milk powder, soya bean meal, tryptone and inorganic nitrogen sources such as ammonium hydrogen carbonate, ammonium chloride, ammonium sulphate, urea, sodium nitrate and potassium nitrate were taken for the study. The respective nitrogen source was added individually at the concentration of 0.05 g/g dry substrate and medium without any of these nitrogen sources was taken as control.

4.5 Effect of metal ions on esterase production

Ten different trace elements (zinc chloride, magnesium chloride, barium chloride, mercuric chloride, cobalt chloride, manganese sulphate, EDTA, zinc sulphate, copper sulphate and calcium chloride) were supplemented individually into the basal medium at the concentration of 0.01 g/g dry substrate. The medium devoid of trace elements was taken as control.

4.6 Effect of Physical parameters on esterase production

Solid state fermentation was carried out to optimize various physical parameters influencing esterase production. These included incubation temperature (10, 20, 30, 40, 50, 60, 70 and 80^{0}C), initial pH (3, 4, 5, 6, 7, 8, 9, 10 and 11) and initial moisture content of the substrate (55, 60, 65, 70, 75, 80, 85 and 90 %).

4.7 Effect of organic solvents on esterase production

To study the effect of organic solvents on esterase production, eight different hydrocarbons were used for experimentation. They were hexadecane, heptane, tetradecane, octane, dodecane, nonane, paraffin liquid and hexane. The selected hydrocarbons were incorporated individually into the production medium with substrate at the concentration of 0.05 ml/g dry substrate and the medium without any of these hydrocarbons was taken as control.

5. Statistical Analysis

The results obtained in the present study were subjected to relevant statistical analysis using Microsoft Excel 2005. The tests for significant differences were analyzed using One-way Analysis of Variance (ANOVA).

Results and Discussion

1. Effect of various triglycerides on esterase production

The effect of various triglycerides on esterase production was tested and resulted in Figure 1. The result showed that castor oil has its maximum influence on esterase production (33.771 U/ml). It was >39.1% production than the least producing olive oil (13.214 U/ml) supplied medium. Mostly triglycerides are found to be an inducer of lipase/esterase production. In the present study, all the supplied lipidic sources significantly ($p<0.0001$) influenced the esterase production. This is quiet important since it proved that the castor oil is cheapest oil, could be used as a convenient lipid/carbon source for the industrial level production of esterase. This study gives significant results and it was correlated with the studies by Bora and Kalita (2008) on the effect of vegetable oils for lipase production by Thermophilic *Bacillus* sp. DH4. The present study also correlated with the lipase production by *Rhizopus* sp. BTNT-2, where influenced positively by olive oil, tributyrin, coconut oil and caster oil (Bapiraju et al., 2005).

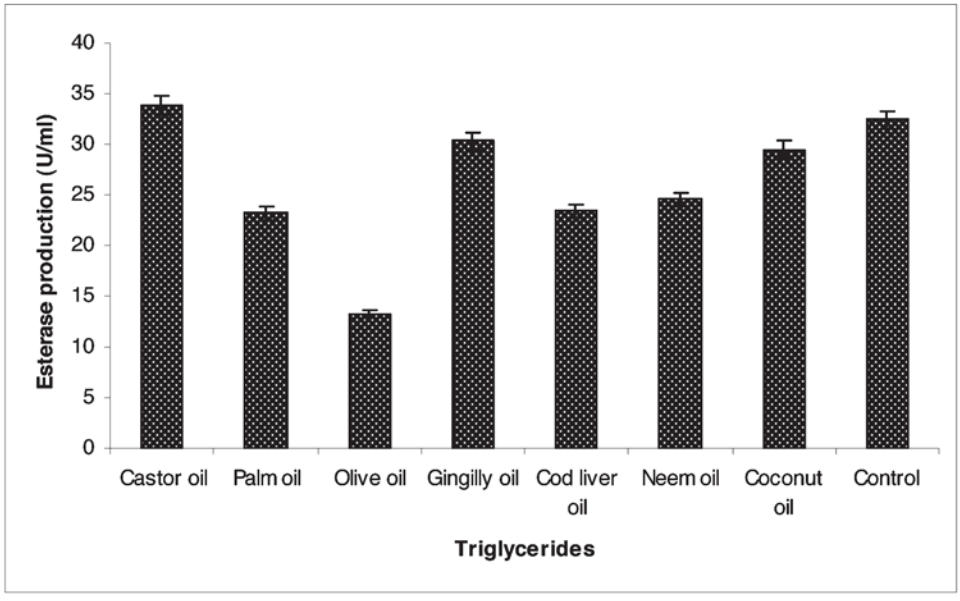

Figure 1. Effect of different triglycerides on esterase production

2. Effect of surfactants on esterase production

The effect of various surfactants on esterase enzyme production was studied under solid state fermentation and it is represented in Figure 2. The result indicated that like triglycerides, all the tested surfactants positively influenced the esterase production over the control (11.39 U/ml) and maximum amount of enzyme production was found in Tween 80 supplied medium (44.505 U/ml) and also the variation in esterase production between the tested surfactants was statistically more significant ($p<0.0001$). Surfactants are known to improve the bacterial enzyme production by enhancing the surface tension between water and nutrients (Reddy et al., 1999). The addition of surfactants to the culture medium increases the lipolytic enzyme production may be due to the alteration of cell permeability leading to increase in protein secretion or its effects on cell bound enzymes (Costas et al., 2004). The result on the present study supports the effect of surfactants on the lipase production by *Aureobasidium pullulans* AP39, where induced highly by Tween 80 (Kudanga et al., 2007). This study also correlates with the esterase activity of *Geobacillus* sp. HBB-4, highly induced by Tween 80 (Metin et al., 2006).

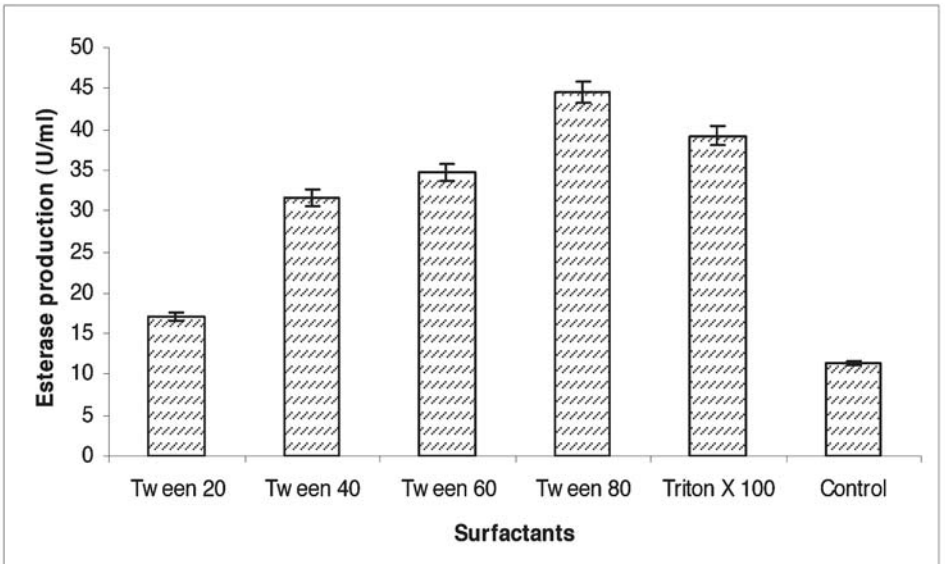

Figure 2. Effect of different Surfactants on esterase production

3. Effect of sodium chloride on esterase production

Figure 3 represents the effect of sodium chloride on esterase production through solid state fermentation. Among the tested concentrations of sodium chloride, maximum amount of enzyme production was recorded at 4% NaCl added medium which gave 77.571 U/ml. The lowest (10.12 U/ml) amount of esterase was found in 11% NaCl added medium. The variation in esterase production between tested concentrations of NaCl was statistically more significant ($p<0.0001$). The result indicated that increasing concentration of NaCl over 4% significantly lowered the enzyme production. Sodium chloride is one of the prime factors, because the bacterium was isolated from the gut of marine fish *S. longiceps*, it is very much important for the maintenance of osmotic balance. Nowadays, the halophilic enzymes have greater influence in the industrial level including detergent industry. This study supports the previous studies of Karpushova et al. (2005) on the halophilic esterase by *Bacillus* sp. associated with sponge *Aplysiana aerophoba.* This was also supported with the halophilic lipase production by marine *Pseudomonas* sp. MSI057 (Seghal Kiran et al., 2008).

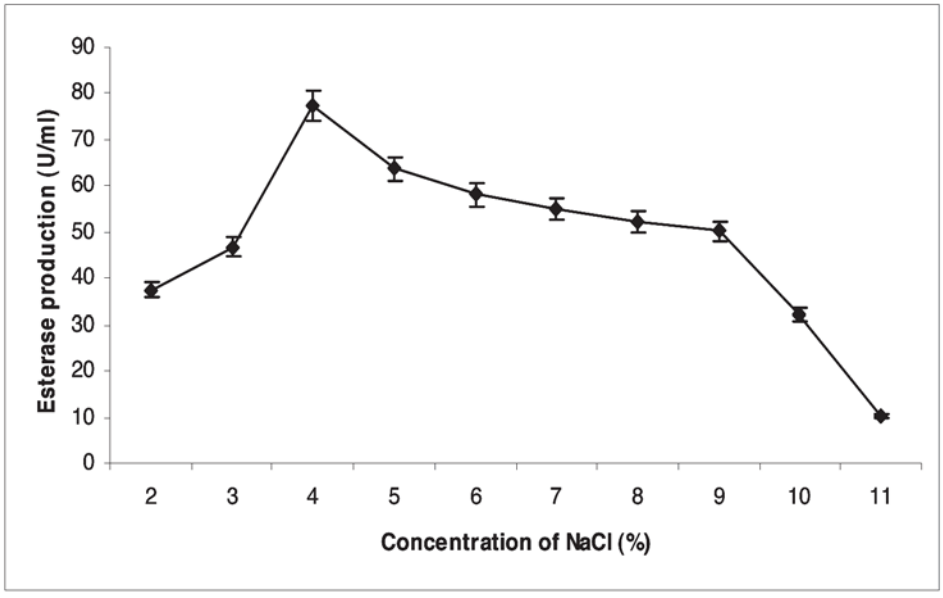

Figure 3. Effect of different concentrations of NaCl on esterase production

4. Effect of nitrogen sources on esterase production

Nitrogen source is another one important factor in any fermentation process for successful production of biomolecules. The experiment on effect of nitrogen sources on esterase production under solid state fermentation revealed that among the tested nitrogen sources, maximum (50.285 U/ml) amount of enzyme production was recorded in ammonium hydrogen carbonate added medium. But the lowest (17.455 U/ml) amount of enzyme production was observed in tryptone added medium. The change in esterase production between all the tested nitrogen sources was statistically more significant ($p<0.0001$). In the present study mostly the inorganic nitrogen sources greatly influenced the esterase production over organic nitrogen source and control (31.3 U/ml) (Figure 4). Like this study, Rodriguez et al. (2006) reported that lipase production by *Rhizophus homothallicus* under SSF was highly influenced by urea supplied medium. Similarly Lima et al. (2003) reported that the lipase production by *Penicillium aurantiogriseum* was high in ammonium sulphate added medium.

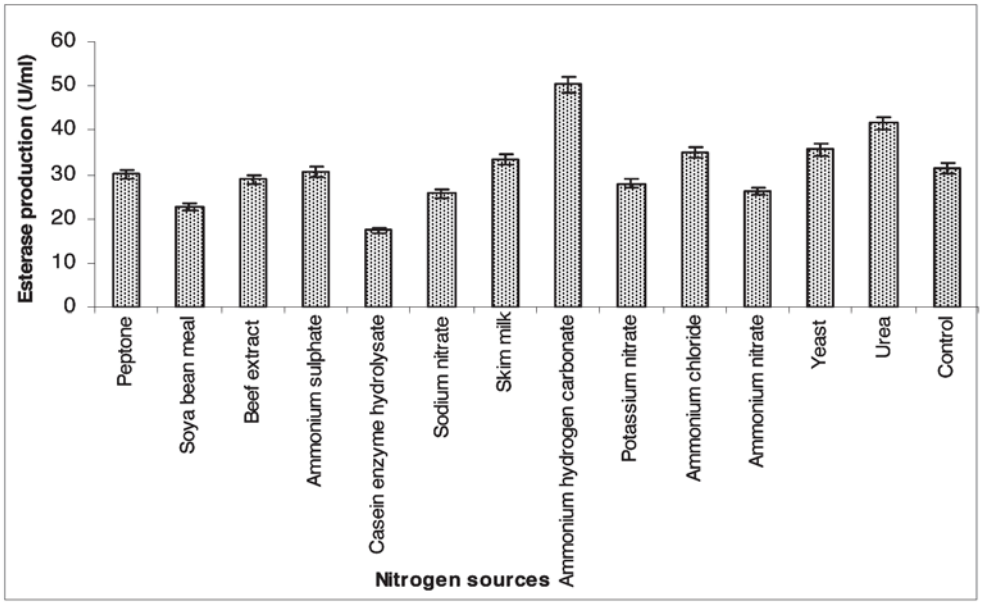

Figure 4. Effect of different nitrogen sources on esterase production

5. Effect of carbon sources on esterase production

In SSF, it is necessary to supply ready sugar for better growth and esterase production and the present results on the effect of supplementary carbon sources on esterase enzyme production is provided in Figure 5. Among the tested carbon sources, maximum (73.702 U/ml) amount of enzyme production was recorded in maltose supplemented medium than the control (65.116 U/ml) and other carbon sources. The lowest (52.647 U/ml) enzyme production was observed in fructose added medium. The differences in esterase production between tested carbon sources was statistically more significant ($p<0.0001$). However, in the present study, the variation found in esterase production by supplying different carbon sources is limited, because the maximum esterase producing carbon source maltose gave only 11% higher yield than control. The results of the present study were correlated with the studies by Alkan et al. (2007), they stated that the lipase production by *Bacillus coagulans* under SSF using melon wastes was highly influenced by maltose and starch. Similarly, this result is in agreement with the studies of Mahanta et al. (2008) on lipase production by *Pseudomonas aeruginosa* PseA with maximum production in maltose supplied medium. Also the present study correlate with the higher amount of lipase production by *Candida rugosa* on 1.5% maltose supplied medium (Rao et al., 1993).

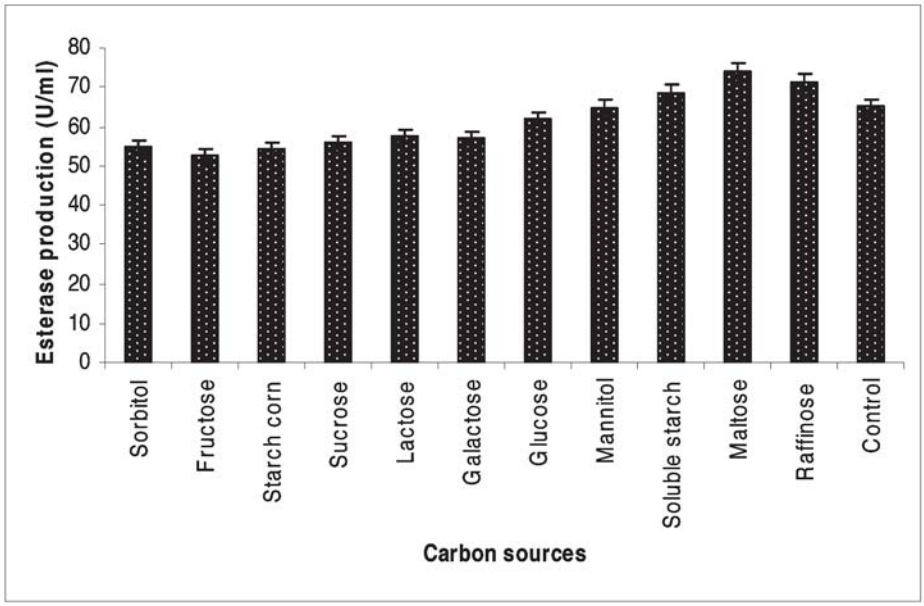

Figure 5. Effect of different carbon sources on esterase production

6. Effect of metal ions on esterase production

Trace elements or metal ions are considered as another basic growth factor in bioprocessing of biomolecules. As for the influence of metal ions on the esterase production, the esterase activity was high (39.12 U/ml) in $CaCl_2$ supplied medium over the control (6.6 U/ml) and other trace elements (Figure 6). The variation in esterase production between tested trace elements was statistically more significant ($p<0.0001$). This is because the enzyme including esterase requires calcium ions for their stable and active structures. They may strongly bind with the specific binding sites on the surface of the molecules. The significant result obtained from the present study supports the previous studies of Alkan et al. (2007) on the positive effect of $CaCl_2$ on lipase production by *Bacillus coagulans*. This study also correlate with the $CaCl_2$ enhanced esterase activity by *Geobacillus* sp. HBB4 (Metin et al., 2006) and $CaCl_2$ induced lipase production by *Bacillus* sp. (Mohd Shariff et al., 2007).

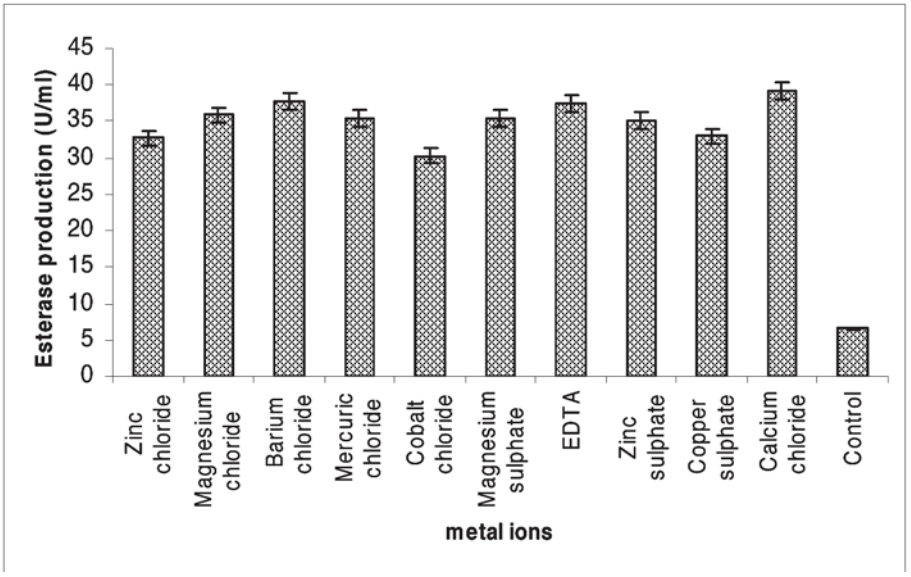

Figure 6. Effect of different metal ions on esterase production

7. Effect of initial pH on esterase production

The pH of the fermentation medium is an essential physical factor, and it may change during the fermentation process. Because the substrate used in the SSF process usually have less buffering. Here the experiment on the effect of pH on esterase production under SSF revealed that this bacterium produces the enzyme over wide pH ranges from 7-9, it was high (58.84 U/ml) at pH 9 and it was alkaline in nature (Figure 7). The variation in esterase production between tested initial pH was statistically more significant ($p<0.0001$). This study supports with the finding of Castro-ochoa et al. (2005) on higher lipase production by *Bacillus thermoleovorans* at pH 9-10. Similarly, the present study is in agreement with the reports of Bora and Kalita (2008) and Kundanga et al. (2007) on higher lipase production in alkaline medium by *Bacillus* sp. DH4 and *Aureobasidium pullulans*, respectively.

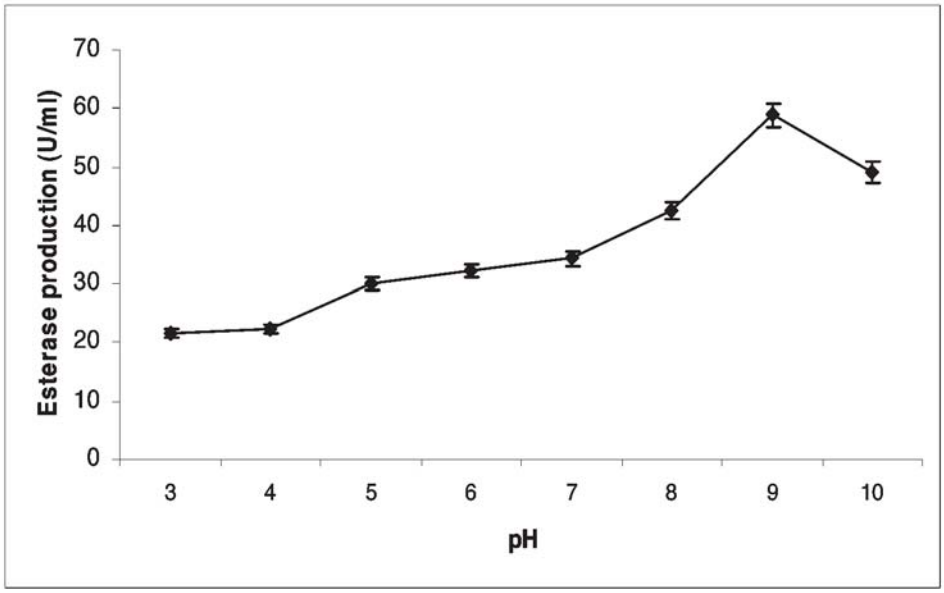

Figure 7. Effect of initial pH on esterase production

8. Effect of temperature on esterase production

Esterase production at various medium temperatures showed that 60 ^{0}C gave maximum (65.679 U/ml) synthesis when compared to any other tested temperature (Figure 8). When increasing the temperature over the optimum level, the enzyme synthesis decreased. Because temperature is one of the important criteria in fermentation process particularly the bioprocessing of enzymes. At this high temperature, the enzyme production was maximum and this enzyme was found to be thermophilic in nature. Nowadays, the thermophilic enzymes have great deal in industrial level application and this esterase can be used in the production process occurred in high temperature. The present study on esterase production at high temperature is in agreement with the previous studies on higher temperature influence on lipase/esterase production by *Bacillus* sp. DH4 at 60-700C (Bora and Kalita, 2008), *Thermoanaerobacter tengcongensis* at 50-60^{0}C (Zhang et al., 2003) and *Bacillus* sp. at 70^{0}C (Mohd Shariff et al., 2007).

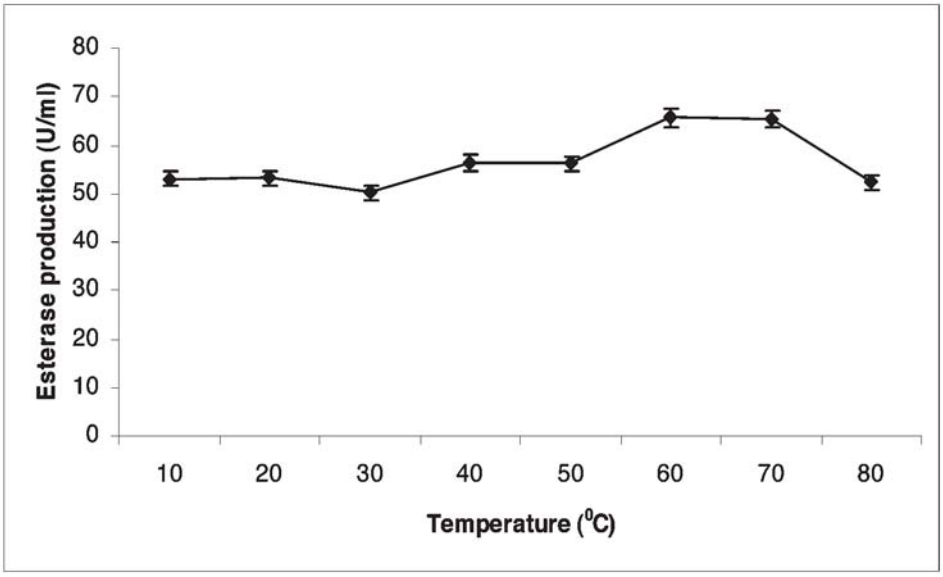

Figure 8. Effect of incubation temperature on esterase production

9. Effect of initial moisture level on esterase production

The initial moisture level in the solid media is a critical factor in solid state fermentation. In the present study, it has played an important role in solid state esterase production with groundnut oilcake by *B. circulans.* The optimal (63.5 U/ml) enzyme production was registered at 75% moisture level. At low moisture level of 30%, minimum enzyme production was noticed (Figure 9) and the difference in moisture content was significantly ($p<0.0001$) varied on esterase production. Similar to the present study, Toke et al. (2007) reported that the esterase production by *Aspergillus* strains was at optimum initial moisture level of 60-67%.

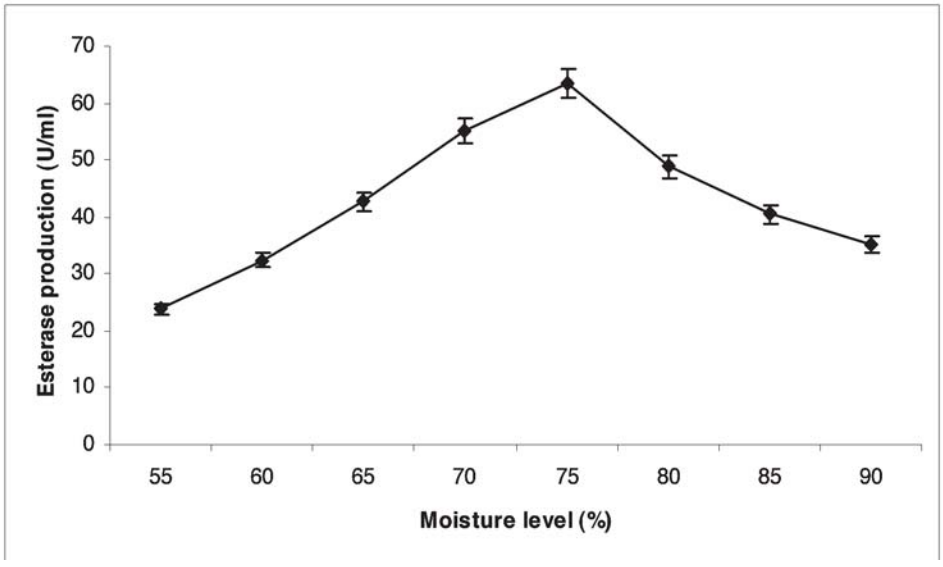

Figure 9. Effect of initial moisture content on esterase production

10. Effect of organic solvents on esterase production

Screening, isolation and identification of novel microbial strains able to tolerate in organic solvents were unattended until the last few years. In the present study the effect of various organic solvents on the esterase production revealed that hexane (41.26 U/ml; 102%), paraffin liquid (42.54 U/ml; 104%) and nonane (43.81 U/ml; 107%) were found to increase the esterase production over the control (40.83 U/ml; 100%) and the effect was very less when compared to control without organic solvents (Figure 10). The variation in esterase production between tested organic solvents was statistically more significant ($p<0.0001$). But the bacterium produced stable esterase over the organic solvents. Hence, this enzyme could be used in the organic synthesis of industrially important fatty acids. Previous studies also find significant results with present study. Studies by Torres et al. (2005) proved that esterase production by *Bacillus licheniformis* S-86 was high in organic solvents such as Butan-1-01 and propan-2-01.

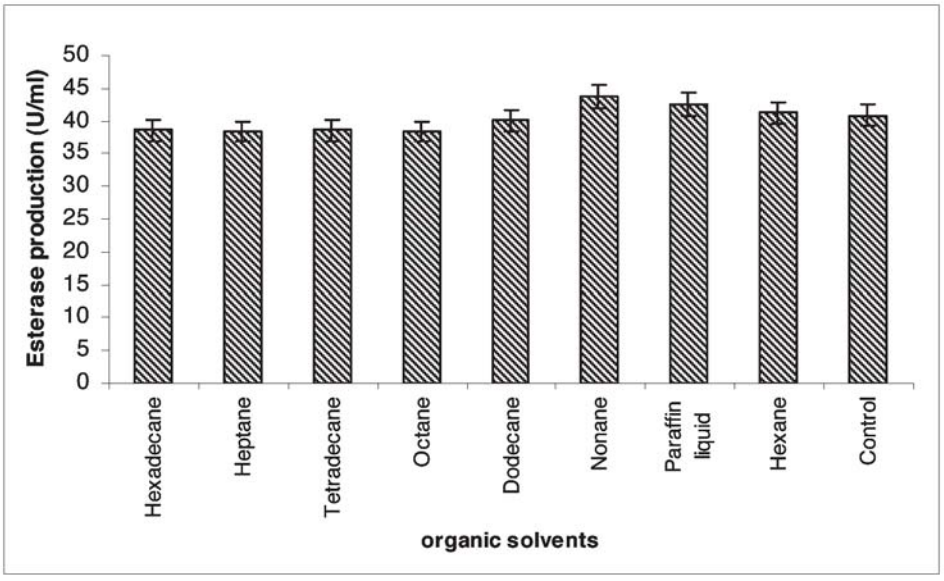

Figure 10. Effect of different organic solvents on esterase production

Conclusion

The present study reports the production of novel halophilic esterase by *Bacillus circulans* isolated from the gut of marine fish *Sardinella longiceps* using cheap agro industrial substrate ground nut oil cake. Owing the potential to use cheap nutrients for maximizing esterase production, it can reduce the production cost. Besides this, the halotolerance and organic tolerance, this esterase can be used for many industrial purposes.

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