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Bioconversion of Disposed Marine Waste into Lipase Enzyme by *Streptomyces fungicidicus* RPBS-A4

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A B S T R A C T

Extracellular lipase production by *Streptomyces fungicidicus* RPBS-A4, previously isolated from the Bay of Bengal has been investigated in submerged fermentation using different proportions of disposed marine wastes such as fish, shrimp and crab as nitrogen source. Among the tested, shrimp waste supported the maximum lipase production. The effect of carbon sources on lipase production revealed that lactose aided the higher lipase production than any other tested carbon source and a concentration of 1.5% lactose registered as optimum to enhance the lipase production. Among the nitrogen sources tested, lipase production was high in peptone added medium when compared to other nitrogen sources, and its optimum concentration recorded was 1.5%. Partial characterization of crude enzyme revealed that pH 9.0 and 45°C temperature were optimum for maximum lipase activity.

Introduction

Lipases, (triacylglycerol acyl hydrolases, EC 3.1.1.3), are natural catalysts of the hydrolysis of triacylglycerols into di- and monoacylglycerols, fatty acids and glycerol at an oil–water interface, a phenomenon known as interfacial activation (Schmidt *et al*., 1998). However, under certain conditions, they are also able to catalyze synthetic reactions (Carvalho *et al*., 2006). The most reported of the reactions carried out by these enzymes are hydrolysis, acidolysis, alcoholysis, aminolysis, esterification and inter-esterification (Saxena *et al*., 2003). Currently, lipases are a popular choice as a biocatalyst because they can be applied to chemo, regio and enantio selective hydrolyses and also in the syntheses of a broad range of compounds (Jaeger and Eggert, 2002). These enzymes are considered to have great potential as biocatalysts in numerous industrial processes, such as the synthesis of food ingredients (Macedo *et al*., 2003), their use as additives to detergents (Liu *et al*., 2009) and to obtain enantiopure drugs and other refined products (Wang *et al*., 2009). In the chemical industry, they are used for the production of surfactants and detergents, to resolve the racemic mixtures and for the treatment of residues that are rich in oils and

fats. In the health sector they are used in medicines, diagnostics, cosmetics and antibiotics (Hasan *et al*., 2006). In the food industry, lipases are used to synthesize emulsifiers such as mono-and diglycerides (Kittikun *et al*., 2008) and for the production of lipids with high levels of polyunsaturated fatty acids (Reshma *et al*., 2008). They are also used for the development of flavors (Salah *et al*., 2007), the maturation of cheese (Dupuis *et al*., 1993) and sausage meat, among others. Furthermore, lipases have an important application in the field of bioenergy, particularly for the production of biodiesel (Park *et al*., 2006), which is an expanding sector, given the worldwide concern with the use of renewable energy.

The marine industry generates nearly 50–60% of the total weight of fish as waste contains enormous amount of protein (35–50%) and chitin (15–25% of dry weight) which are considered as major environmental pollutants due to its uncontrolled dumping (Islam *et al*., 2004). Production cost of lipase is strongly influenced by expensive medium components like nitrogen, carbon sources such as fatty acids, triglycerides and sugars or complex polysaccharides like glycogen and surfactants. Bioconversion of these disposed fish waste materials has been proposed to be a safer waste treatment option that will solve environmental problems, but also decrease to a large extent the production costs of using microbial enzymes (Liu *et al*., 2003). Interestingly, fish wastes provide an excellent source for microbial growth, which can be exploited in producing various metabolites (lysine, enzymes, etc.) (Coello *et al*., 2000; Vazquez *et al*., 2006, 2008).

Most of the marine actinomycetes are exploited for production of antibiotics but the studies on novel enzyme production from marine actinomycetes is still in infant stage (Bull *et al*., 2000). Only about 2.0 per cent of the world's microorganisms have been tested as enzyme sources. Streptomyces are the most important industrial microorganisms because of their capacity to produce numerous bioactive molecules, particularly antibiotics and enzymes (Lam, 2006).However many studies were made on production and purification of lipase, no attempt has been made on lipase from marine actinomycetes through bioconversion of marine wastes. Hence in the present study, an attempt was made on production and characterization of lipase from *Streptomyces fungicidicus* RPBS-A4 by utilization of marine wastes.

Materials and Methods

Growth Curve for *Streptomyces fungicidicus* RPBS-A4

A set of ten 100 ml conical flasks containing 20 ml of starch casein broth were inoculated with *Streptomyces fungicidicus* RPBS-A4. All the flasks were incubated at 30°C and 120 rpm on orbital shaker. One flask was harvested everyday till the tenth day. The biomass was separated from the nutrient medium by centrifugation and washed with distilled water. The biomass was transferred on a filter paper and kept for drying at 55°C. The dry weight was determined after 24 hrs.

Time Scale Analysis of *Streptomyces fungicidicus* RPBS-A4

Time scale analysis was performed in order to determine the fermentation period required for maximum production of lipase. The fermentations were carried out using production medium (Bindiya and Ramana, 2012) supplemented with 1% olive oil at 30ºC for 168 hrs. Samples were withdrawn every 24 hrs and extracted. The extract was

assayed for lipase activity (Jensen, 1983; Chandan and Sahani, 1964).

Preparation of marine waste based media

Marine wastes such as fish, shrimp and crab were collected from disposed sites in Vadarevu near Chirala coast of Andhra Pradesh (India). Five kg of each waste were packed in separate insulated polythene bags and stored at -20ºC until use. The disposed waste included heads, viscera, shells and tails. The waste collected were minced by a mixer grinder, mixed with water (500 g/l) and heated at 100ºC for 20 min. After heat pre-treatment, insoluble material was removed by centrifugation (9390 g for 10 min) and the supernatant was stored at 4ºC until use. Supernatants supplemented with different proportions (0%, 25%, 50% and 75%) of standard medium (SM) were used as media for bacterial growth. The SM was composed of 17 g/l casein peptone, 5 g/l yeast extract and 2.5 g/l glucose, pH 7.4 (Ben Rebah *et al*., 2008).

Comparison of enzyme production in different media combinations

The marine isolate *Streptomyces fungicidicus* RPBS-A4 was grown in 3 different media combinations (fish, shrimp and crab waste) to test best growth supporting media and best enzyme producing media. The media were prepared in 100 ml conical flasks contained 50 ml of supernatant derived from marine waste (fish, Shrimp and crab) supplemented with different proportions (0%, 25%, 50% and 75%) of standard medium (SM) and pH was adjusted to 7.0.The flasks were inoculated with 8mm disc of inoculum grown on starch casein agar medium for 72 hrs. The flasks were incubated at 30°C for 96 hrs at 120 rpm.The crude enzyme was extracted at regular time intervals and lipase activity was determined titrimetrically. The extent of growth supported by all the above stated media were also examined because the production of lipase is directly related to growth of organism. The biomass was separated from the medium by centrifugation and washed with distilled water. The biomass was transferred on a filter paper and kept for drying at 55°C. The dry weight was determined after 24 hrs.

Media Optimization for Lipase Production

Growth conditions and media composition were optimized in separate experiments.

Effect of inoculum size on lipase production

The isolate *Streptomyces fungicidicus* RPBS-A4 was inoculated on shrimp waste based medium, as this medium gives relatively fast growth and enzyme activity. Submerged fermentations were carried out by inoculating cutting discs of 5, 6, 7, 8 and 10 mm diameters in different sets. Fermentation's were carried out at 30ºC for 96 hrs. Samples were withdrawn after 96hrs and centrifuged; the supernatant collected was assayed for lipase activity and the dry weight was also determined.

Effect of Carbon sources on lipase production combined with substrate

For the identification of suitable carbon source for lipase production, different carbon sources such as glucose, lactose, sucrose, maltose, groundnut oil, neem oil, coconut oil and palm oil were tested individually at the level of 0.5%. Subsequently, the maximum lipase producing carbon source was further optimized by varying its concentrations such as 0.25%, 0.5%, 0.75%, 1.0%, 1.25% 1.5% and 1.75% in the production medium.

Effect of Nitrogen sources on lipase production combined with substrate

The effect of nitrogen sources on lipase production was tested by adding different nitrogen sources such as ammonium sulphate, ammonium carbonate, peptone, tryptone, beef extract and yeast extract. After screening the maximum lipase yielding substrate, it was further optimized by varying concentrations (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, and 4.0%).

Effect of incubation temperature on lipase production

To determine the effect of temperature on enzyme production, fermentation was carried out at different incubation temperatures ranging from 20ºC to 55ºC. The samples were withdrawn after 96 hrs and the extracts were assayed for lipase activity.

Effect of p^H on Lipase Activity

To determine the optimum initial medium p^H for actinomycetes growth and enzyme production, the production medium p^H was adjusted from 4.0 to 11.0 using buffering agents (0.1M/L), flasks cultured in a incubator-shaker (120 rpm) at 40^{o}C and crude enzyme extract was prepared and lipase activity determined.

Effect of agitation speed on Lipase Activity

To determine the optimal agitation speed for peak enzyme activity, the *Streptomyces fungicidicus* RPBS-A4 was cultured in an orbital shaking incubator at 40°C at varying agitation speed from 60-140 rpm. The fermentation process was terminated after 96 hrs and crude enzyme extract was prepared and lipase activity determined.

Results and Discussion

Growth Curve for *Streptomyces fungicidicus* RPBS-A4

The appearance of biomass in the medium started after 24 hrs and increased successively. The growth of *Streptomyces fungicidicus* RPBS-A4 appeared in the form of small beads in clear transparent medium which is a characteristic feature of *Streptomycetes* (Williams *et al*., 1989). The number and size of beads increased as the incubation time increased. The biomass showed a progressive increase till 6th day of incubation but later on the growth was not much. The organism seems to enter the stationary stage after 6th day of incubation. The growth of the isolate is reported in the form of dry weight and graphically presented in Figure.1.

Time Scale Analysis for *Streptomyces fungicidicus* RPBS-A4

Production was terminated in a set of flasks after every 24 hrs and the broth was harvested to determine the amount of enzyme produced. The accumulation of enzyme increased till 96 hrs of incubation. Further incubation did not support the increase in productivity of lipase. As the number of bacterial cells goes on increasing the number of enzyme molecules shall increase, therefore high biomass accumulation will result in good enzyme yield. The production of lipase is reported by Vishnupriya *et al*., (2010) in 24 hrs, Aly *et al*., (2012) observed the production in 72 hrs and sathya priya *et al*., (2012) in 96 hrs by *Streptomycetes*. Maximum accumulation of enzyme in our case was observed in 96 hrs of incubation as also reported by Selvam *et al*., (2013) for extracellular production by *Streptomyces variabilis*. Further incubation resulted in loss of enzyme activity. Aysel *et*

al., (2014) reported maximum production in 120 hrs. The decrease of the enzyme activity during the stationary phase may be explained by the detrimental effects of acidic pH or some by-products formed in the medium. Presence of proteases may also breakdown the enzyme present in the medium. The lipase activity during seven days period is graphically depicted in Figure. 2.

Selection of Medium Combination

One problem regarding enzyme production on a large scale is the production cost. A possible alternative for reduction in production cost will come about with the preparation of medium with low cost nitrogen sources. Viewing this, in the present study, an attempt has been made to reduce the production cost using a cheap nitrogen source (marine wastes). Various media combinations were tried for the production of lipase. All the media combinations checked were giving varying enzyme yield and different biomass accumulation. Among all nonsupplemented samples tested as growth media, only supernatants derived from shrimp and fish waste based media showed highest enzyme activity. Interestingly, shrimp waste based medium gave the highest growth yield (24.85mg/ml) and exhibited the maximum lipase activity (12.91 U/ ml). In this case, growth yield and lipase activity values exceeded those obtained with the standard medium (SM) (18.23mg/ml and 10.83 U/ ml).The supplementation of supernatants with various proportions of SM influenced the growth and lipase activity. In all experiments, the maximum growth was obtained upon adding 75% SM and the highest growth was obtained for shrimp waste based medium. These results find support with the previous report of Ben Rebah *et al*., (2008) on lipase production by *Staphylococcus xylosus*. They inferred that shrimp waste induce maximum lipase production than other fish wastes. This study also supports the previous findings of Ellouz *et al*., (2003) and Souissi *et al*., (2008) on the possible use of fish wastes as nitrogen source for bacterial growth and protease production.

All these media combinations were also checked for stimulating the growth of *Streptomyces fungicidicus RPBS-A4*. The maximum biomass was produced in shrimp waste based media supplemented with 75% standard medium. This medium also stimulated high enzyme production but lesser than non supplemented shrimp waste based media. The results of above comparison are graphically represented in Figure.3.

Effect of Inoculum Size on production of Lipase

The optimisation of fermentation process for industrial application also requires suitable medium for inoculum development. The time required for growth of inoculum should be as less as possible. This can be achieved by selection of a medium which supports fast growth of the organism and also initializes the machinery for the formation of desired product (lipase) in the organism. Starch casein medium is reported to induce lipase production as well as growth of *Streptomycetes* (Srilekha Mishra and Nibha Gupta, 2014). The inoculation of *Streptomyces fungicidicus* RPBS-A4 was done by cutting discs of varying sizes, 5, 6, 7, 8 and 10 mm diameter with the help of a cup borer. The enzyme production was measured for all the flasks. The enzyme yield was increased in the flasks on increasing the inoculum size. The increase in all the parameters was progressive from 5 mm disc containing flask to 8 mm disc

containing flask, but there was no remarkable increase beyond this. The optimisation of industrial processes is based on utilizing minimum resources and getting maximum outputs therefore the marginal increase beyond 8 mm diameter is not considered significant and 8 mm disc is picked up as the optimum size of inoculum. The graphical representation of effect of varying inoculum size is shown in Figure. 4 for lipase production and growth response in terms of biomass accumulated.

Effect of carbon sources on the production of lipase

Carbon is a major component of the cell and the rate at which a carbon source is metabolized can often influence the formation of biomass or production of metabolites Stanbury *et al*., (1997). In the present study, 8 different carbon sources, glucose, maltose, lactose, sucrose, groundnut oil, neem oil, coconut oil and palm oil were tried to obtain suitable and low cost carbon source for maximum lipase production. Among different carbon sources tried, lactose gave maximum yield (14.16 U/ml) in the media followed by neem oil (10.83 U/ml). Lactose is also reported as an inducer for the enzyme produced by *Streptomyces variabilis* NGP3 Selvam *et al*., (2013). The results of the effect of carbon sources checked in shrimp waste based medium are presented in Figure.5.

Increasing concentrations of lactose also increased the enzyme productivity but till a certain limit beyond which there was no substantial increase. There was an increase in the productivity of lipase from 5.83 U/ml in 0.25g/100ml of lactose to 15.41 U/ml of enzyme in the fermented broth containing 1.5% of lactose concentration but further increase did not affect the production. This must be due to the highest lactose concentration reached which the organism can bear. Lactose is reported to give high yields of lipase by earlier researchers also. The results of quantitative effect of lactose are shown in Figure. 6.

Effect of nitrogen sources on the production of lipase

Nitrogen sources including organic and inorganic sources play an important role in the synthesis of enzymes. Organic nitrogen sources can supply many cell growth factors and amino acids, which are needed for cell metabolism and enzyme synthesis. In general, microorganisms afford high yields of lipase when an organic form of nitrogen is used. The inorganic nitrogen sources showed an inhibitory effect on lipase production (Sarkar *et al*., 1998). In the present study among six different nitrogen sources, peptone gave maximum yield (14.58 U/ml). The results of the effect of nitrogen sources checked in media are presented in Figure.7. The nitrogen source peptone favored the lipase production in the case of *Aspergillus. sp* Cihangir and Sarikaya, (2004), *Staphylococcus sp*. Lp12 pogaku *et al*., (2010).

All the nitrogen sources tried peptone was found to be the best among them. Therefore its quantitative effect on lipase production was studied. Production of enzyme increased from 11.66 U/ml to 15.83 U/ml in the medium with increase in peptone concentration from 0.5 g/100ml to 1.5 g/100ml but further increase substantially decreased the production. The literature survey shows that nitrogen content in the media used for the production of lipase ranges from 0.3% to 3% and our results are also falling in the same range (Alok kumar *et al*., 2013). The results are shown in Figure.8.

Effect of Temperature on production of Lipase

The production of lipase was found to be maximum at 37°C as also reported by Aly *et al*., (2012) for lipase production by *Streptomyces exfoliates*. Sirisha *et al*., (2010) observed maximum lipase production from a non filamentous organism *Staphylococcus* at 36°C. The results are presented in Figure. 9. The temperature optima were determined and highest enzyme production was observed at 40°C. Many of the earlier researchers also have reported the production of enzyme between 30°C to 35°C Selvem *et al*., (2013). Thermophilic organisms are also reported to produce lipase in considerable concentrations having high optimum temperature of 55°C as optimum for lipase production from *Streptomycetes* isolated from marine sediments Sathyapriya *et al*., (2012).

Effect of p^H on production of Lipase

A broad range of p^H was tested for optimisation of lipase production by *Streptomyces fungicidicus* RPBS-A4. There was no production as well as growth observed at p^H 4.0 and 5.0 but the flasks having p^H 8.0 and 9.0 exhibited substantial production of lipase. The production was further reduced on p^H 10.0 and 11.0 but the overall picture exhibited by the organism indicates the preference of alkaline p^H range with maximum at p^H 9.0. There are earlier reports on production of lipase at alkaline p^H, Aly *et al*., (2012) exhibited maximum lipase production at p^H 6.0, Nadia *et al*., (2001) observed at p^H 7.0 and Sathyapriya *et al*., (2012) observed at p^H 9.0. Selvam *et al*., (2013) also observed *Streptomyces variabilis* producing lipase between p^H 9.0 to 9.5. The results are presented in Figure.10.

Effect of Agitation Rate on production of Lipase

The production of lipase from *Streptomyces fungicidicus* RPBS-A4 was studied on a range of agitation rates. The highest yield of lipase was observed at 120 rpm on shrimp waste based medium. The results are presented in Figure. 11. Lipase production in the flasks incubated at 60 and 80 rpm was exhibited very poor yield but those incubated at 120 and 140 rpm was ample enough. Selvam *et al*., (2013) have reported optimum lipase production at 120 rpm.

Fig.1 Growth curve of *Streptomyces fungicidicus* RPBS-A4.

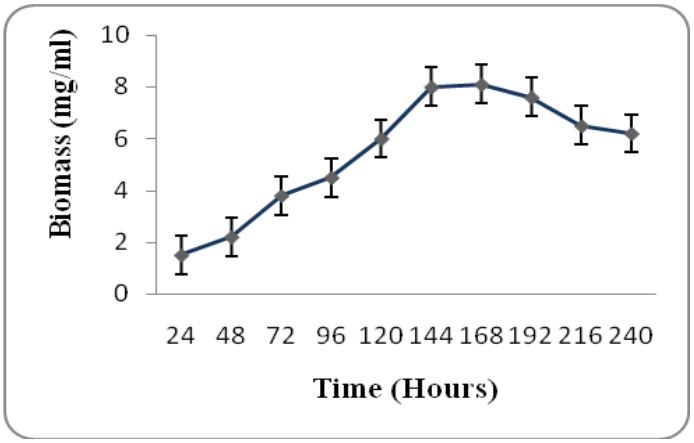


Fig.2 Production of Extracellular lipase at different time intervals by *Streptomyces fungicidicus* RPBS-A4.

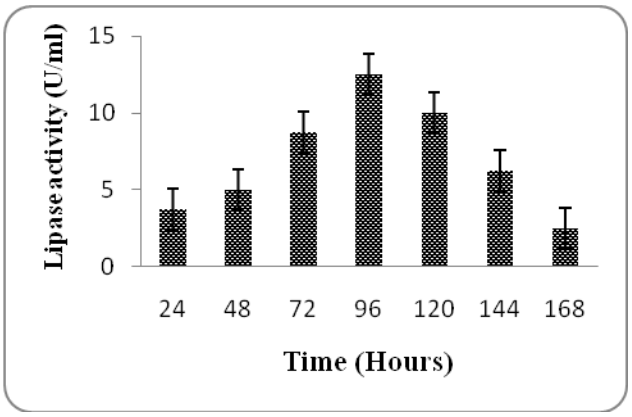


Fig.3 Effect of different media combinations on lipase production and biomass yield

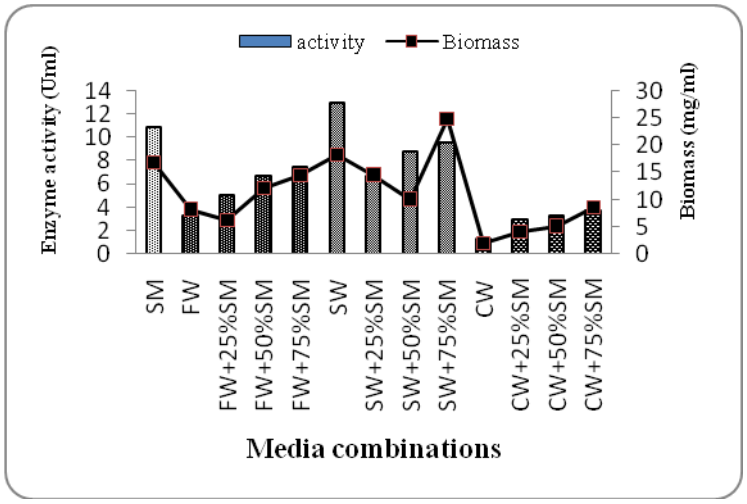


Fig.4 Effect of inoculum size on lipase production

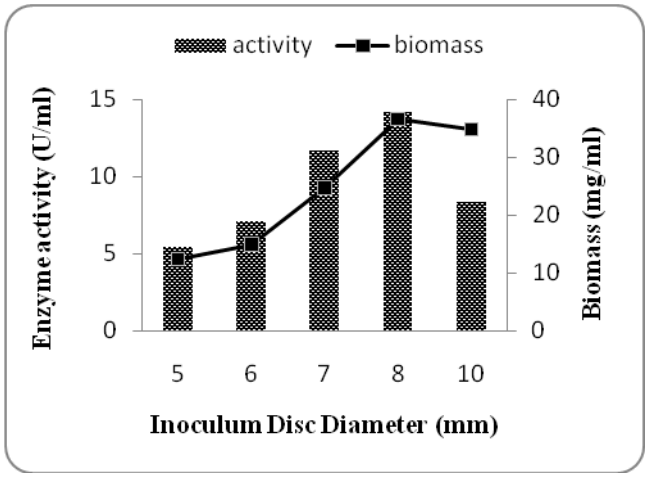


Fig.5 Effect of carbon sources on lipase production

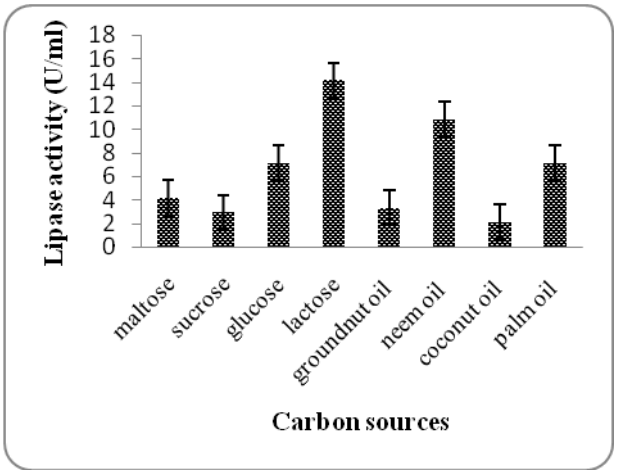


Fig.6 Effect of concentration of carbon source on lipase production

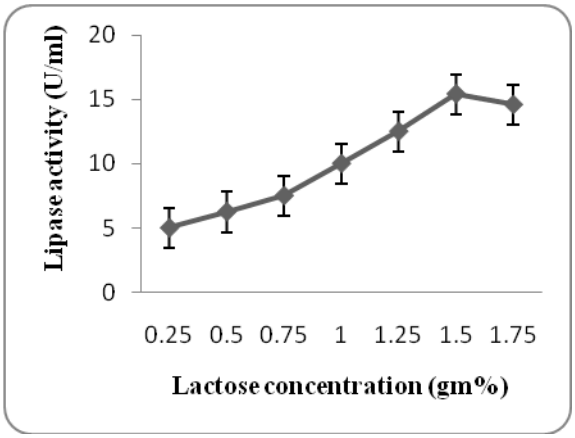


Fig.7 Effect of nitrogen sources on lipase production

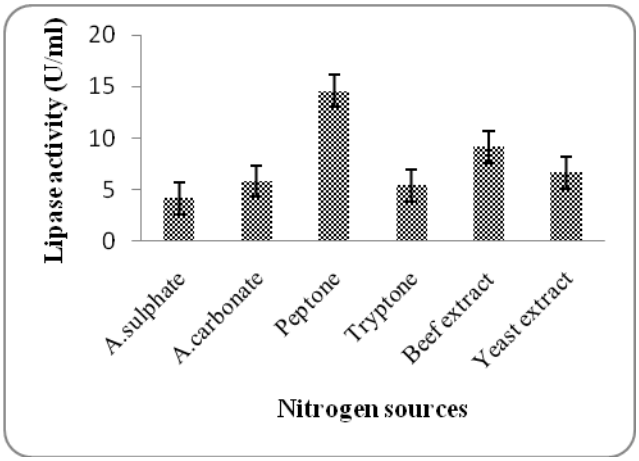


Fig.8 Effect of concentration of nitrogen source on lipase production

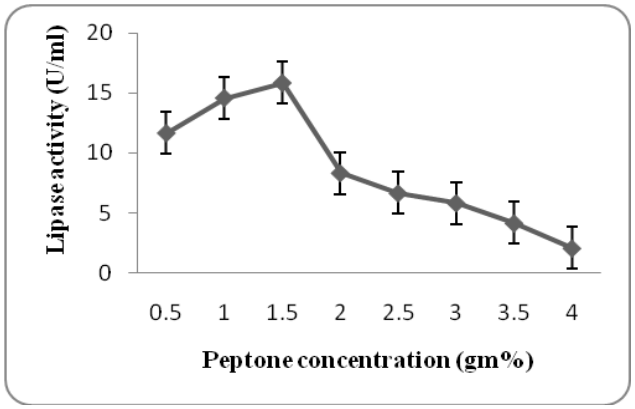


Fig.9 Effect of temperature on lipase production

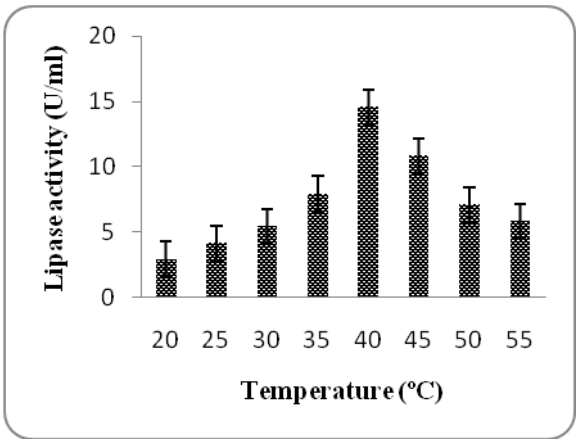


Fig.10 Effect of pH on lipase production

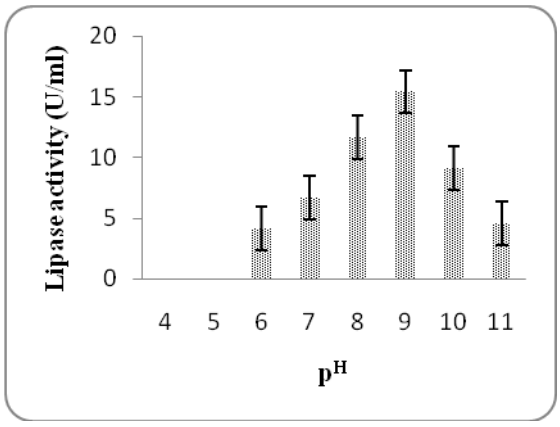
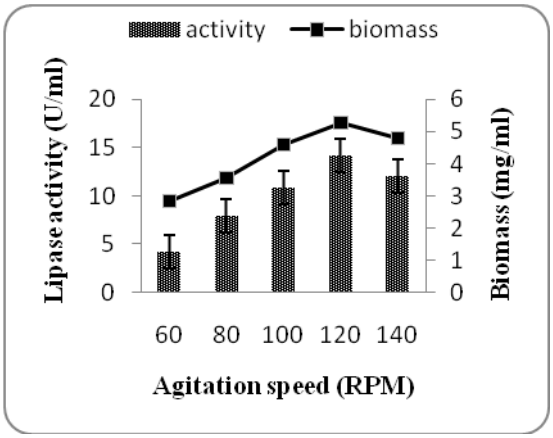


Fig.11 Effect of agitation rate on lipase production



In conclusion, the biomass increase was observed continuously for 10 days but there was a progressive increase till 6th day which is associated with enzyme accumulation, this also indicates that the organism moves to its stationary phase where the increase in enzyme accumulation ceases. The decrease in the enzyme level in later days must be due to the proteolytic activities going on in the medium and decrease in the number of surviving cells in the decline phase of organism's life cycle which can compensate the loss. Time scale analysis showed that maximum activity of enzyme obtained till 96 hrs of incubation. The isolate *Streptomyces fungicidicus* RPBS-A4 was able to produce lipase on a wide range of carbon and nitrogen sources. The best was lactose among the pure carbon sources and shrimp waste based medium among marine wastes. Presence of peptone exhibited the enhancement in lipase activity and biomass. The scaling up of process requires transition of medium ingredients to crude sources. The crude sources like shrimp waste and fish waste gave good yields which is again beneficial for industrial purposes.

The ingredients we found to be beneficial are lactose in 1.5% concentration, peptone in 1.5% concentration. The temperature optima were determined and highest enzyme production was observed at 40°C. Production of lipase at alkaline p^H 9.0 exhibited maximum lipase production. The production of lipase was studied on a range of agitation rates. The highest yield of lipase was observed at 120 rpm on shrimp waste based medium. The results indicate that shrimp waste can be used as economically available marine wastes for industrial production process. Use of marine wastes for industrial production of metabolites not only reduces the production cost but also solves the problem of disposal of tonnes of marine wastes produced every year in a fish supplying country like India.

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