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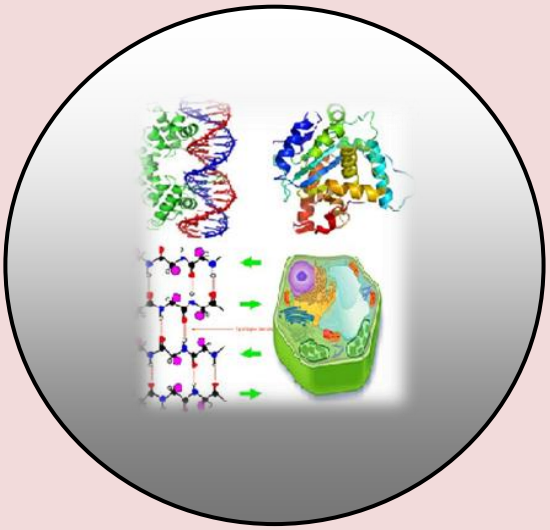

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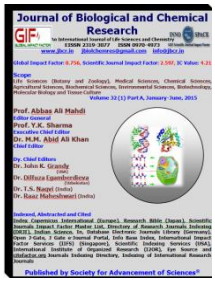

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Production of Lipase Enzyme by *Aspergillus oryzae* using Fishery Waste

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ABSTRACT

Aspergillus oryzae is an aerobic, filamentous and a non-pathogenic fungus. Solid substrate fermentation using fungi is one of the economic modes of lipase production. Different media components such as nitrogen, carbon and lipids were optimized. Moreover, the physico chemical parameters like pH and temperature were also optimized for effective production of lipase. Among the experiments tested, tuna waste used for nitrogen source on lipase production proved to be more efficient than the other fish waste using Aspergillus oryzae. Fructose supported the maximum lipase production in Aspergillus oryzae. The effect of lipid source tested for lipase production was high in cod liver oil using Aspergillus oryzae. The pH and temperature optima for lipase production were 6 and 40°c in Aspergillus oryzae.

Key words: Fish waste, Aspergillus oryzae, Lipase and Solid Substrate Fermentation.

INTRODUCTION

Fish processing industries in India generates 2 million metric tons of fishery waste (Nurdiyana and Mazlina, 2009). Solid waste which represents 20-60% of the initial raw material contains various kinds of residues (whole waste fish, fish head, guts etc) (Awarenet 2004). Solid waste includes heads, viscera, frames and skin proved to be a great source of 58% protein dry matter and fat (19% D.M) and it contain trace amounts of minerals such as nitrogen, phosphorus, magnesium, sodium, potassium, calcium, iron, zinc, ammonium, manganese and copper (Ramakrishna et al. 2013). These minerals support the growth of microbes as they act as cofactors for various metabolic activities. Myristic, palmitic and stearic acids are the important saturated acids present in fish. Fish oil with higher level of polyunsaturated fatty acids is helpful for human health (Kim, B. S. and Hou, C.T. 2006). However, these solid wastes are not utilized properly and impose threat to the environment by causing infectious diseases to the human beings. Hence, the fish waste with nutritional value may be used for the production of industrially important enzymes.

FUNGAL ENZYMES

Lipases are generally produced from microorganisms like bacteria and fungi. Certain species of fungi viz *Rhizopusrhizo podiformis*, *Rhizomucor pusillus*, *Aspergillus niger*, *Penicillium restrictum*, *Rhizopus oligsporous*, *Rhizopus delemer* and *Aspergillus oryzae* (Cordova et al., 1998, Kamini et al., 1998, Gombert et al., 1999, ul-Haq et al., 2002, Mahadik et al., 2002, Christen et al., 1995, Ohnishi et al., 1994).

Among these strains, *Aspergillus niger* is found to produce significant quantities of enzyme and is regarded as generally regard as safety (GRAS) by Food and Drug Administration (FDA). Whereas maximum lipase activity of solid substrate was observed using *A. niger* NCIM 1207 (Mahadik et al., 2002).

Aspergillus oryzae is an aerobic, filamentous and a non-pathogenic fungus. In the beginning, the colony is white, later yellowish-greenish. Conidiophores are hyaline and mostly rough walled. Several isolate are mostly uniseriate, others mainly biseriate. Conidia are large and smooth to finely roughen. The optimal growth temperature is 32-36 ^{O}C. *A. oryzae* is primarily found in China and Japan, where it is used in the fermentation of certain foods. Outside this area, the fungus may be periodically found in soil or on decaying plant material. Only few reports claim that *A. oryzae* has been isolated from areas of temperate climate (two from continental Europe according to Domsch et al). A reason for this may be the relatively high optimal growth temperature of the fungus. The extremely sporadic occurrence in these areas may thus reflect a poor ability of *A. oryzae* to establish itself in temperate zones. For more than 2000 years, *A. oryzae* has been used in the Orient to produce koji, a complex enzyme preparation for the production of soy-sauce, miso and sake. In Europe, *A. oryzae* has been used for baking and brewing. *A. oryzae* and its enzymes are accepted as constituents of food. Hence, this study aimed to use the fungus *Aspergillus oryzae* for lipase production.

Several lipase producing fungal strains have been used for lipase production by SSF. Solid state fermentation process can be done in absence or near absence of free water under controlled conditions. Most of the industrial enzymes are produced from this fermentation process. It has more advantages such as high volumetric productivity, low energy consumption, natural habitat for bacteria and fungal culture, physical support, suitable value addition and cost effective.

MATERIAL AND METHODS

SAMPLE COLLECTION

Fish waste samples such as sardine fish waste, tuna fish waste, shrimp wastes and squid waste were collected from fish processing industries in and around Tuticorin.

PREPARATION OF MEDIA

Potato dextrose agar media was used to culture *Aspergillus* oryzae. Apporoximately, 5g of Potato dextrose agar (PDA) powder was suspended in to 100ml of distilled water. Then, the medium was stirred until completely dissolved and undergo sterilization by autoclaving for 15 minutes at 121 ^{O}C. After that, the medium was cooled to ambient temperature before poured into conical flask. The media was then stored at 4°c for further use.

DEVELOPMENT OF *ASPRGILLUS ORYZAE*

Aspergillus oryzae was purchased from kamni biotech laboratory, Nagerkoil Tamilnadu, India. The culture was grown in Potato dextrose agar (PDA) slant at 28 ^{O}C for 7 days. Subcultures were maintained for further use.

CALCULATION OF LIPASE ACTIVITY

One unit of enzyme is defined as no of µmol of fatty acid released per ml of sample.

µmol fatty acid/ml subsample = *$\frac{\text{(ml NaOH for sample} - \text{ml NaOH for blank) xN x1000}}{5}$

Where N is the normality of the NaOH titrant used (0.05 in this case) and 5ml is volume of reaction mixture used.

PRODUCTION OF LIPASE BY SSF

The media optimization experiment was started by cultivating the fungi in enrichment media containing different nitrogen sources (sardine, shrimp, tuna and squid), different carbon sources (glucose, fructose, sucrose, lactose and maltose) and different lipid sources (coconut oil, gingerly oil, palm oil, cod liver oil and castor oil). Each sample was taken in 250 ml flask, then the culture was inoculated in to each flask. The culture flasks were incubated at 25 ^{O}C for 7 days. After incubation 100 ml of distilled water was added to each culture flask and left it 1hr. The culture containing enzyme was filtered through what man filter paper no. 1. The filtrate was used for further titrimetric determination of lipase assay [Praphan Pinsirodom and Kirk L. Parkin, 2001].

PROTEIN WAS ESTIMATION BY USING LOWRYS METHOD

EFFECT OF pH

Optimum pH for lipase activity was determined by using different pH buffers (pH4 and pH5 for acetic acid and sodium acetate, pH6 for citric acid and sodium citrate and pH7 and pH8 for NaH_2Po_4 and Na_2HPo_4) respectively.

EFFECT OF TEMPERATURE

The effect of temperature on lipase activity was studied by incubating the medium containing nitrogen source, carbon source and phosphate buffer at various temperature (30, 35, 40, 45, 50 and 55 °C) respectively.

RESULTS AND DISCUSSION

EFFECT OF NITROGEN SOURCES ON LIPASE PRODUCTION

A variety of nitrogen sources were tested to estimate their capability to support lipase production. The nitrogen content of above said four fish waste materials (Sardine, Shrimp, Tuna and Squid fishes for 1 to 8 gms) with ammonium sulfate as control. Here, Tuna fish waste has supported high nitrogen sources which give maximum lipase activity, when compared to other fish waste material. Figure 1 represents the fungi showing the lipase activity in nitrogen sources of different fish waste material. The test for lipase production from tuna fish waste has high efficacy at 8g concentration (78.3U/ml). However, squid has shown lowest lipase activities of 40.1U/ml.

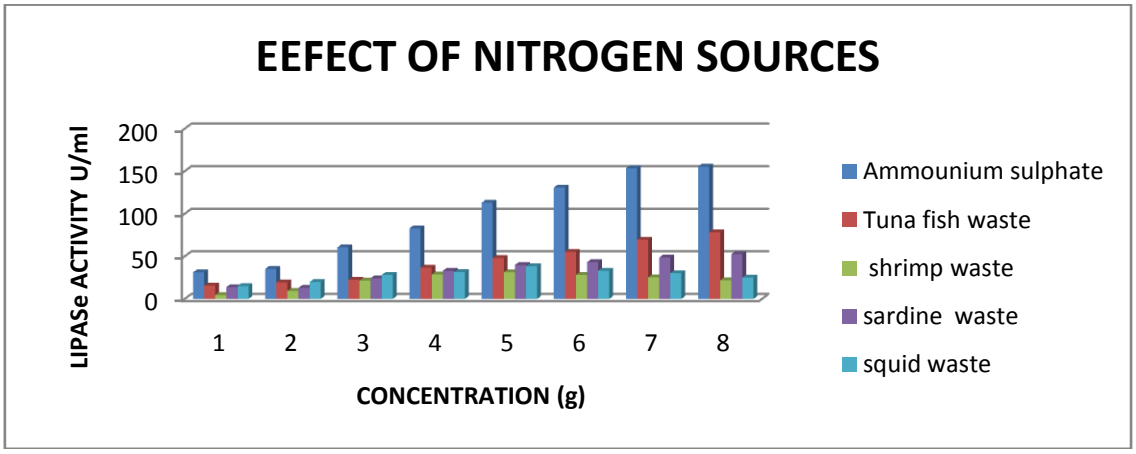


Figure 1. Effect of Nitrogen Sources (NH_3SO_4, Tuna Fish Waste, Shrimp Fish Waste, Sardine Fish Waste and Squid Fish Waste) Using *Aspergillus oryzae*.

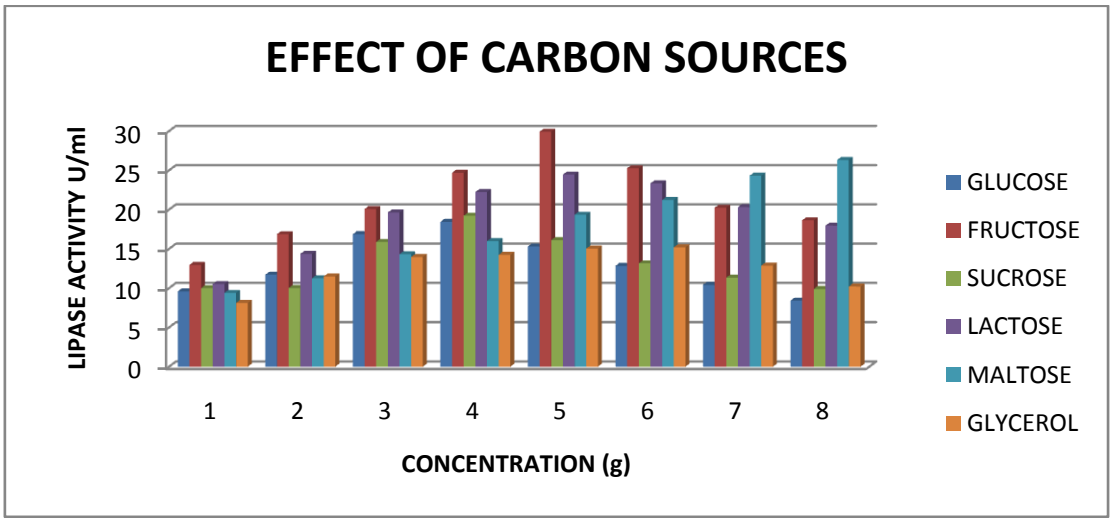


Figure 2. Effect of Carbon Source (Glucose, Fructose, Sucrose, Lactose Maltose and Glycerol) using *Aspergillus oryzae*.

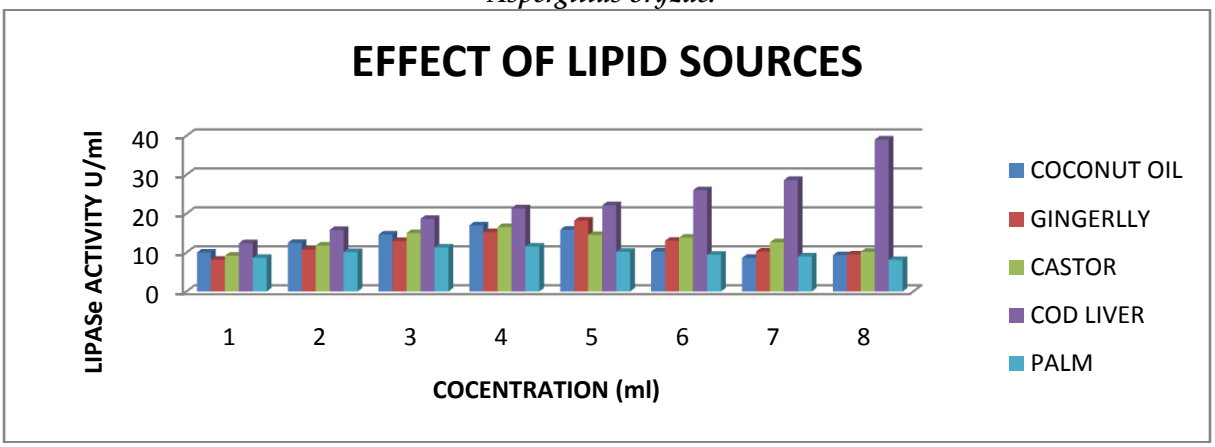
EFFECT OF CARBON SOURCES ON LIPASE PRODUCTION

Based on Nitrogen source result, the maximum lipase production activity was observed in Tune fish waste. Hence, the tuna fish waste has been chosen for the carbon source analysis for lipase production. Different carbon sources such as glucose, fructose, sucrose, maltose and glycerol (1gm to 8gm) were used to assess lipase production. From these carbon sources, the highest lipase activity was found in fructose in the concentration of 6gm (29.9U/ml). The lowest lipase activity (15 U/ml) was observed in glycerol.

EFFECT OF LIPIDS SOURCES ON LIPASE PRODUCTION

From the carbon source test resulted the best carbon source was fructose and the best nitrogen source was Tuna fish waste. So we have chosen the tuna fish waste with fructose carbon source. These were tested with lipids for producing more lipase. Five different lipid sources were tested for lipase production. These are coconut oil, gingerly oil, castor oil, cod liver oil and palm oil. Among the lipid sources, the highest lipid content was shown in cod liver oil at 8 gm (39 U/ml) concentration. However, palm oil lowest lipase activities of 11.5 U/ml at 4g concentration.

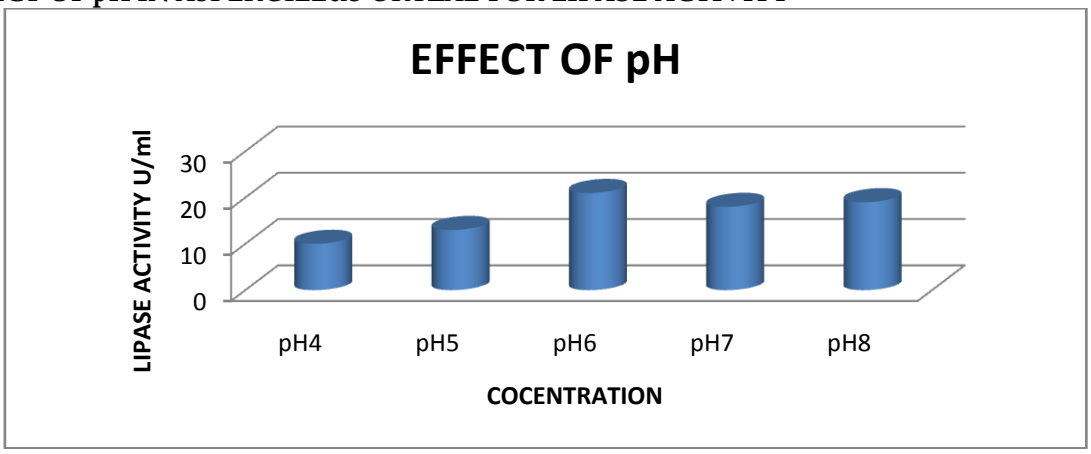
Effect of Lipid Source (Coconut Oil, Gingerly Oil, Castor Oil, Cod Liver Oil and Palm Oil) using *Aspergillus oryzae.*



EFFECT OF pH ON LIPASE PRODUCTION ACTIVITY

The pH stability of the purified lipases was also investigated by measuring the residual activity at pH values ranging from 4.0 to 8.0. Apart from moisture, pH and water activity also exert a decisive influence on microbial metabolism. The tested results were revealed that Optimum pH for lipase activity was determined by using different pH buffer (pH4 and pH5 –acetic acid and sodium acetate, pH6- citric acid and sodium citrate and pH7 and pH8- NaH_2PO_4 and Na_2HPO_4) in the reaction. The assay was carried out individually at various pH values (4 to 8). Figure 7 describes that the highest lipase produced at acidic in nature, that was recorded in pH 6 (21.6 U/ml) followed by pH 8 and pH 7. The lowest lipase produced by pH 4.

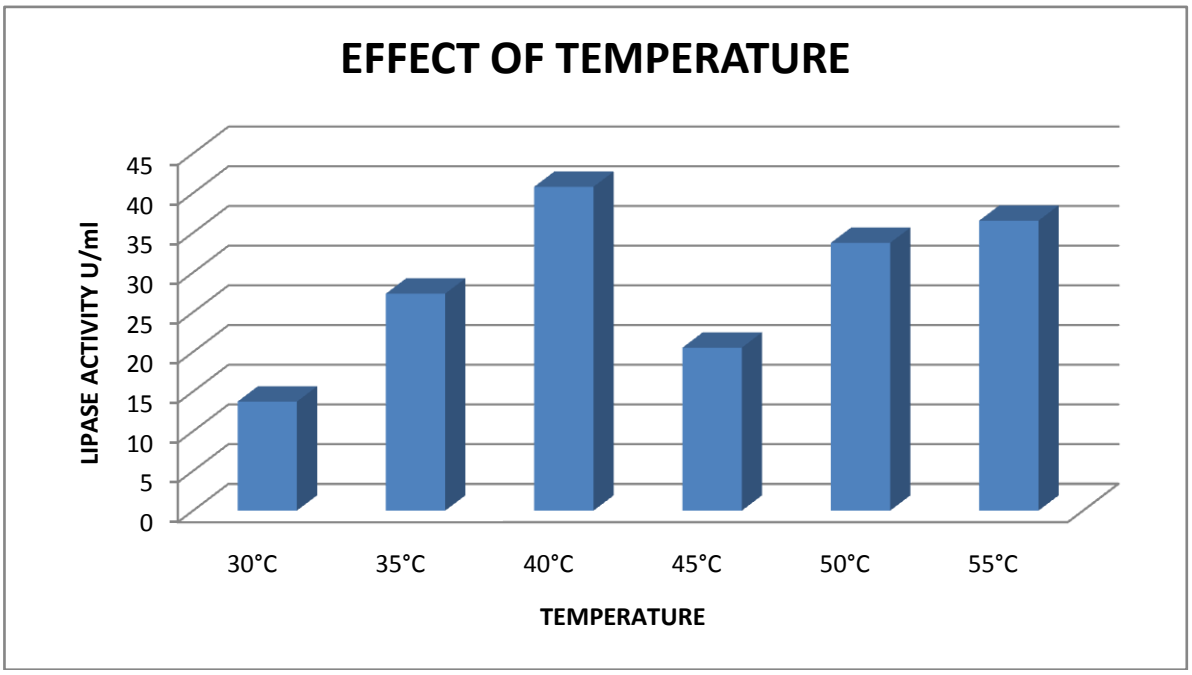
EFFECT OF pH IN *ASPERGILLUS ORYZAE* FOR LIPASE ACTIVITY



EFFECT OF TEMPERATURE ON LIPASE PRODUCTION

The effect of temperature on lipase activity was studied by incubating the medium containing nitrogen source, carbon source and phosphate buffer at various temperatures (30, 35, 40, 45, 50 and 55 ^{O}C). Maximum lipase activity was found at 40 ^{O}C (40.8 U/ml) Followed by 55 ^{O}C, 50 ^{O}C, 35 ^{O}C and 45 ^{O}C. The lowest temperature was produce lipase activity at 30 ^{O}C.

EFFECT OF TEMPERATURE IN *ASPERGILLUS ORYZAE* FOR LIPASE ACTIVITY



DISCUSSION

Nitrogen sources (organic and inorganic) used to supplement the culture medium greatly influences lipase production (Ramos-Sánchez et al., 2015). In many cases, only the waste is sufficient to meet the microbial needs with respect to nitrogen for growth and enzyme synthesis (Ramos-Sánchez et al., 2015). The lowest nitrogen sources which produce low lipase activity in squid fish waste when compared to other fish waste material. It is due to the fish waste material were collected from different environment with different seashore areas. Similar values of lipase production were also reported by Di Luccio et al. (2004) who cultivated *Penicillium simplicissimum* in SSF using soybean cake and olive oil as inducer, the maximum lipase activity (21 U ·g-1) being attained after 94 hours of cultivation at 27 ^{O}C and with 4% olive oil. Sun and Xu (2008) also studied lipase production with the fungus *Rhizopus chinensis* using flour and wheat bran as substrate (3:2 w/w). The effects of nutritional factors such as Carbon, Nitrogen, Lipids, Minerals and Substrates on lipase production were studied. All experiments were carried out in triplicate and average values are presented in tables.

Carbon is the 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). Mahanta et al. (2008) and Rao et al. (1993) who reported that presence of maltose in the growth media enhanced the lipase production by *P. aeruginosa* and *C. rugosa.* Rodriguez et al., (2006) reported that that fructose was the most suitable carbon source for maximum lipase production by *Rhizopus homothllicaus*. Different carbon sources have been reported as best for maximum lipase production using different bacteria and fungi. However, few studies have been published that are aimed at the production of lipase enzyme using glycerol as a carbon source. Lipases are often inducible enzymes, and a similar observation has been reported regarding lipase production by *Serratia rubidaea*, which is induced by gingerly oil (Immanuel et al. 2008). Similarly, Bora and Kalita (2002) found that lipase production by Bacillus sp. DH 4 was higher in medium supplied with vegetable oils.

Microbial lipases are of wide interest because they are highly selective, stable and substrate specific (Treichel et al., 2010). The substrate and reaction specificities of lipases surpass those of any other known enzyme and the application potentials are limitless (Gandhi, 1997).

This study was also supported by the positive effect of olive oil, tributyrin, coconut oil and castor oil on lipase production by *Rhizopus* sp. BTNT-2 (Bapiraju et al. 2005). Microbial lipases mostly are inducible. Upon induction, they secrete extracellular enzymes into the surrounding environment. Such inducible extracellular lipases are produced in the presence of inducers such as fatty acids, oils, triacylglycerol, tween, bile salts and glycerol (Yang et al., 2005) although the requirement for sugar as a carbon source in addition to lipids varies with the microorganism (Ayinla et al., 2017).

The change in pH influences the growth of the organism, effects product stability and also induce morphological change in organism and enzyme secretion (Gupta et al., 2003). In consistence many microbial lipases have their optimum activity at pH range of 7-9 as reported by Zhang et al. (2005). Sopuruchukwu et al. (2015) reported optimum lipase production from *Fusarium sp*. when pH of fermentation broth was in acidic range pH 2.5. The initial pH of the medium influences the lipase secretion in different microorganisms (Thomas et al., 2003; Cihangir and Sarikaya 2004, Tan et al., 2004). Lipases with highest activity at pH 7 were also reported in *Candida antartica* (Pfeffer et al., 2006). Similarly, the lipase exhibited moderate activity at pH 7. But in this study the maximum lipase activity exhibited at pH6.

Temperature is the relative lipase activity on enzyme reaction. In general, the temperature required for lipase production corresponds with the growth conditions of the microorganism (Salihu and Alam, 2012). It has been discovered that incubation temperature is a significant controlling factor for enzyme production and increase in temperature tends to favor fungal growth to some extent. In general, the temperature required for lipase production corresponds with the growth conditions of the microorganism (Salihu and Alam, 2012). *A. niger* strains have been reported to be active between 40 and 55 °C (Kamini et al., 1998, Namboodiri et al., 2002). Pera et al. (2006) reported that an optimum temperature for the lipase activity obtained from *A. niger* ATCC MYA-135 was 37 ^{O}C. Hence, the fishery waste based optimized media can be successfully used for the production of *Aspergillus oryzae* lipase using solid substrate fermentation. Similar to our results, an optimum temperature of 30 ^{o}C was reported by Sopuruchukwu et al. (2015) and Maia et al. (1999) for *Fusarium sp*. and Ire and Ike (2014) for *Aspergillus carbonarius*. Supakdamrongkul et al. (2010) have reported an optimum temperature of 25 ^{O}C for lipolytic activity from fungus. An optimum temperature of 40 ^{O}C was reported by Dahiya and Purkayastha (2011) and 45 ^{O}C by Costa and Peralta (1999) for *Penicillium sp*.

CONCLUSIONS

Vital investigation of current literature shows that microbial lipases are one of the most promising enzymes. There has been much development on lipase production by bioprocesses, mainly using solid substrate fermentation such as the screening of high lipase producers, successful substitution of nutritional rich nitrogen sources from different fish waste material with suitable carbon sources. Based on our experiment, we believe that the use of lipases will predominate in the near future since the production of lipases will allow attainment of enzymes with new remarkable characteristics for a specific application. The results discussed in this chapter clearly demonstrate that SSF, as long as the cultivation volume is kept to a very small scale, yields good results in terms of process productivity of lipase enzymes. This study was done with a new promising lipase producing fungal species (*A. oryzae*), which produced extracellular lipase with tuna fish waste and fructose as carbon source. Different physical and nutritional parameters such as lipids, pH and temperature, etc. affected lipase production significantly. Maximum production of lipase was observed at 40 ^{o}C with pH 6.0, when the medium was supplemented with mixture of fructose and cod liver oil as carbon and lipid source. This may increase the potential of tuna fish waste with *A. oryzae* can produce maximum lipase production. Owing the potential of fish waste materials for maximizing lipase production, it can be used for the media formulation for microbiological growth, and subsequently it adds value for waste outputs from processing. Lipase produced by *A. oryzae* is very promising and could be used for industrial purposes. It is hoped that after some scale up studies, our fungal strain *A. oryzae* can be used for mass cultivation and production of large quantities of lipase in SSF. These results, together with the fact that this fungus is generally recognized as a safe microorganism in the food, beverage and pharmaceutical industry, make this process worthy of future investigation.

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