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Optimization of extracellular lipase production in *Colletotrichum gloeosporioides* by solid state fermentation

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Abstract: Twenty two endophytic fungi isolated from oil seeds were screened for their ability to produce extracellular lipolytic enzymes in solid state fermentation. Among them, the most productive strain, identified as *Colletotrichum gloeosporioides* was cultivated for lipase production in solid state fermentation (SSF) using oil-mill residue as a solid support. The elaboration of extracellular lipases from *C. gloeosporioides* on different residual cheap oil substrates was tested. Among these, pongamia oil cake (POC) showed maximum lipase activity (983 U/g dry matter (DM)) followed by coconut oil cake (COC) (925 U/g DM). These two oil industrial residues were chosen for enhanced lipase production. The maximum enzyme yield was obtained when POC was impregnated with CDB (Czapek-Dox Broth) in the ratio of 1:1.5 and with (906 U/g DM). Triton X 100 as the extractant from the fermented solids yielded 1020 U/g DM. The optimum temperature for lipase secretion by *C. gloeosporioides* was found to be 25^0C. Xylose as the carbon source and peptone as the nitrogen source were favorable for the secretion of the enzymes. Tween 60 served as a good lipid substrate for lipase production (1170U/g DM) when POC was the substrate. Amending the POC with Magnesium sulphate as a metallic ion source induced good lipase activity (1240 U/g DM) by *C. gloeosporioides*. The Sunflower oil was found to be best co-vegetable oil substrate to induce the enzyme (2560 U/g DM). Triton X100 served as the surfactant for lipase secretion in *C. gloeosporioides*. Thus, pongamia oil cake can be used as cheap substrate for enhanced extracellular lipase production by *C. gloeosporioides.*

Keywords: Lipases, *Colletotrichum gloeosporioides*, solid state fermentation, pongamia oil cake, coconut oil cake.

Introduction

Lipase (EC 3.1.1.3) hydrolyses triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. Interest in these enzymes has increased markedly over the last decades, in view of their diverse applications in medicines (digestive enzymes), food additives (flavour- modifying enzymes), clinical reagents (glyceride – hydrolyzing enzymes) and cleaners (detergent additives) and for synthesis of biopolymers and biodiesel (Sugiura, 1984; Pandey, 1999a). Lipases can also catalyze reverse reactions, such as esterification and transesterification, in non-aqueous environments, and can show regio- and enantioselectivity. Because of these attributes, lipases find a wide range of applications in industry and fine chemicals (Sharma, 2001). Besides, Solid state fermentation (SSF), which is characterized by microbial growth on moist solids, has proven to be an efficient way to produce enzymes, especially by filamentous fungi, since it provides the microorganisms with the environment akin to their natural habits (Pandey *et al*., 1999a; Durand, 2003). However, like in many applications that demand high enzyme yields, lipase production depends on the cost reduction so as to be economically viable. Solid state fermentation (SSF) provides a viable alternative to produce industrial enzymes at lower costs, (Castilho *et al*., 2000; H¨olker *et al*., 2004) with the advantage of using agro-industrial residues as growth substrates and employing considerably less sophisticated equipment. Oil cakes, in particular, edible oil cakes offer potential benefits when utilized as substrates for bioprocesses. These substrates have been utilized for fermentative production of enzymes and antibiotics etc. (Sumitra *et al*., 2007).

Although SmF (Submerged Fermentation), which is widely used in the enzyme industry, has advantages in process control and good yields of extracellular enzymes, the products in fermentation of beer are relatively dilute and therefore the downstream process releases high volumes of effluents (sewage). As an alternative, solid state fermentation (SSF) has been developed and proved to be an economical way to produce various enzymes including lipases and esterases (Gombert *et al*., 1999; Pandey *et al*., 1999b; Pandey *et al*., 2000; Hoelker *et al*., 2004). Lipase production of various strains by SSF including *Penicillium simplicissimum* (Di Luccio *et al*., 2004), *Penicillium restrictum* (Palma *et al*., 2000; Leal *et al*., 2002), *Penicillium candidum* (Rivera-Munoz *et al*., 1991; Ortiz-Vazquez *et al*., 1993), *Penicillium camembertii* (Ortiz-Vfazquez *et al*., 1993), *Rhizopus* sp. (Macedo *et al.,* 2003), *Rhizopus oligosporus* (Ul-Haq *et al*., 2002), *Rhizopus rhizopodiformis* and *Rhizomucor pusillus* (Cordova *et al.,*1998), *Rhizomucor miehei* (Uvarani *et al.,* 1998), *Mucor miehei* (Ortiz-Vazquez *et al*., 1993),

Mucor racemosus (Bogar *et al*., 2003), *Aspergillus níger* (Olama *et al*., 1993; Mahadik *et al*., 2002; Kamini *et al.,*1998), *Yarrowia lipolytica* (Dominguez *et al*., 2003) or *Candida rugosa* (Rao *et al*., 1993a; Rao *et al*., 1993b; Benjamin & Pandey, 1997) has been reported. An economic analysis of lipase production by *P. restrictum* under solid state and submerged fermentations revealed that for a production scale of 100m^3/year, SSF was more economical than SmF (Castilho *et al*., 2000). Several of these organisms, such as *A. niger* (Mahadik *et al*., 2002) which is considered as GRAS, can be used for large-scale production of enzymes even in the food industry. Agro-industrial residues such as olive oil cake (Cordova *et al*., 1998), soy cake (Di Luccio *et al*., 2004), coconut oil cake (Rao *et al*., 1993a; Benjamin & Pandey, 1997), babassu cake (Leal *et al*., 2002), gingelly oil cake (Kamini *et al.,* 1998), wheat bran (Ortiz-Vazquez *et al.,* 1993; Uvarani *et al.,* 1998; Mahadik *et al*., 2002), rice bran (Rao *et al*., 1993b), almond meal (Ul-Haq *et al*., 2002), sugar cane bagasse (Cordova *et al*.,1998) and castor seed litters (Olama *et al*., 1993) can be used as substrates in the production of lipases by SSF. In some cases, the use of mixed solid substrate, such as coconut oil cake: wheat bran (1:1) (Benjamin & Pandey, 1998), might prove advantageous. With the aid of artificial solid supports, such as nylon sponge (Dominguez *et al*., 2003), liquid agricultural wastes can also be applied as substrates. When cultivated in low-cost solid medium composed of agro-industrial waste, *P. restrictum* produces a pool of hydrolases capable of degrading the most complex organic compounds (Cammarota & Freire, 2006). The use of fermented solids as inocula in solid-state fermentation appears to be a viable alternative, especially for large-scale fermentations where generation of large volumes of spore suspensions is a difficult proposition (Gutarra *et al.,* 2007).

Lipases from many microbes have been extensively studied because of their potential applications. However, lipase production by endophytic fungi has received relatively little attention. In this investigation, the feasibility of production scale- up of lipolytic enzymes using an isolate *C. gloeosporioides* with POC and COC as substrates has been worked out.

Materials and Methods

Twenty two fungal strains in all were tested on solid medium for their ability to produce extracellular lipolytic enzymes. The fungi with the highest lipolytic activity were chosen for the study of lipase production by SSF. Among them, *C. gloeosporioides* was found to be best lipase producer and the same was used in the study. The optimal conditions for the growth and production of lipolytic enzymes were investigated.

Culture conditions

The organism was cultured in 250 mL flasks containing 20g POC or COC as solid support impregnated with a concentrated broth, in a ratio of 1g per 1.5 mL, of nutrient source modified culture medium consisting of KH_2PO_4 – 1.0g, $MgSO_4$ – 0.5g, KCl - 0.5g, $FeSo_4$ – 0.01g, Xylose - 8g, Peptone – 20g, Tween-60 – 15 mL, Triton x 100 – 4 ml and sunflower oil– 25 mL in one liter medium. The initial pH of the medium was adjusted to 6.5 and incubated at 25 °C for 7 days.

Solid State Fermentation (SSF)

Oil cake used as the SSF support was sieved to provide particle sizes between 0.8 and 1.7 mm washed thrice with distilled water and then dried at 80^0C for two days. SSF was carried out as described by Raimbault and Alazard (1980). Oil cake was impregnated with a concentrated culture broth, in a ratio of 1.5 mL /g, and was sterilized at 121^0C for 15 min. The inoculum was added to a number of sterile Erlenmeyer flasks (250 mL) containing 20g solid support was impregnated with CDB broth. After mixing, the flasks were incubated under suitable conditions.

Enzyme extraction

After 7 d incubation, 1% (W/V) Triton X100 with Phosphate buffer 20 mM (pH8) was added to each flask. The flasks were kept in a rotary shaker at 200 rpm for one hour at 25^0C. The ingredients of the flask were subsequently filtered and the culture filtrate was used as the enzyme source. The relative humidity and dry matter were determined by drying 5g of fermented solid matter at 100^0C for 24h.

Lipase assay

Lipase activity was measured spectrophotometrically based on the liberation of copper soaps of free fatty acids (Kwon & Rhee, 1986). An emulsion was prepared by mixing in a homogenizer (19,000 rpm, 10 min. 4^0C), 20g of olive oil and 100 mL of a 0.1 M Tris- HCl buffer (pH 8 at 40^0C), containing 0.25 % PVA and 10 mM $CaCl_2$. Reaction was started at 40^0C by the addition of 100 µL initial enzyme extract to 5 mL emulsion and was allowed to run for 15 min before stopping with 1 mL 6 M HCl. Tubes were then incubated in a boiling water bath for 5 min and fatty acids released were extracted in 5 mL isooctane by vortex for 1.5 min. 0.5 mL copper acetate-pyridine was added to 2.5 mL organic phase recovered by centrifugation (5000 ×g, 15min). After vortexing, for 15 min, the absorbance was measured at 715 nm in a spectrophotometer. A blank was also run side

by side. Each value presented in the figure represents the mean and standard deviations. A calibration curve was plotted against oleic acid using a range of concentrations from 0 to 10 µM. One unit of activity is defined as the amount of enzyme releasing 1µ mol of fatty acid per minute under the test conditions.

Statistical analysis

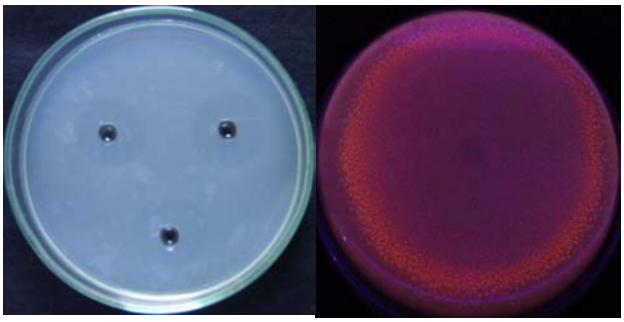
All the experiments were carried out in triplicate, the values presented in the graphs are those of the mean of three independent experiments and the error bars indicate standard deviation.

Results and Discussion

Current developments in biotechnology are yielding new applications for enzymes. Solid State fermentation (SSF) holds tremendous potential for the production of enzymes. Selection of a substrate for the enzyme production in a SSF process depends upon several factors, mainly related to the cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In this investigation oil extraction industrial residues as substrates were studied. The nature of the solid substrate employed is the most important factor affecting SSF processes and its selection depends upon several factors.

Twenty two wild fungal species associated with oil-rich seeds, were screened. The fungi were isolated by adopting the usual procedure. The fungal species so recovered were screened for the production of extracellular lipase production. Among them, *C. gloeosporioides* was identified as the best lipase producer and the same was chosen for further study (Fig.1). Investigations on the selection of suitable substrates for SSF have centred on the industrial residues in view of their potential advantages for filamentous fungi, which are capable of penetrating into the hardest of these solid substrates, aided by the presence of turgor pressure at the tip of the mycelium (Ramachandran *et al*., 2004). A strain of *C. gloeosporioides* was found to be the best in secreting an alkaline lipase under submerged and solid state fermentation, and was able to hydrolyze a wide range of oils and lard (Colen *et al*., 2006).

Fig. 1. Determination of extracellular lipase production by C. gloeosporioides



A. Tributyrin agar diffusion method (hydrolysis of tributyrin by lipase action observed by clear zone) and B. Rhodamine-B fluorescent dye method (appear opaque and pink colored lipase zone visible on irradiating plates with UV light

In the first place, *C. gloeosporioides* was screened for extracellular lipase production under solid state fermentation with different oil cakes as the substrates (Fig.2). Pongamia oil cake was found to be the best substrate for this purpose yielding 983 U/g DM followed by coconut oil cake yielding 925 U/g DM. Coconut oil cake as a potential substrates for SSF process in *Candida rugosa* was reported earlier (Rao *et al*.,1993a; Benjamin & Pandey, 1997; 1998). Taking advantage of supporting high enzyme elaboration, these two oil cakes were subsequently tapped for their ability under varied physico-chemical culture environments. This is the first report using pongamia oil cake (POC) and coconut oil cake (COC) for SSF by *C. gloeosporioides*. The other oil cakes tried out were that of ground nut, gingelly and castor. But they were not much promising comparatively. Two other oil cakes, neem and madhuca have been poor inducers of the enzymes. From the fact that pongamia and coconut oil cakes favoured lipase elaboration shows that these oil cakes have some ingredients which promote enzyme synthesis and further studies are required to find out the active principle in the oil cakes contributed to enhance enzyme synthesis.

Regarding the effect of moisture content on lipase secretion, it was observed that 1:1.5 (substrate: water w/v) was favorable (Fig. 3). Similar trend was reported by Kamini *et al*., (1998) with *Aspergillus niger* but the observation was not in consistent with that of Mahadik *et al*., (2002) who observed the maximum lipase activity with SOB medium (synthetic oil based medium) at a 1:2.5 ratio, when wheat bran was used as substrate. Therefore it is evident that the enzyme activity is subject to the water holding capacity of the substrate and that it varies from substrate to substrate.

The efficacy of the extraction medium is of crucial importance. Triton X100 appears to be the best extractant (Fig. 4). The yield of the enzyme with the extractant is in the order of 1020 U/g DM with pongamia oil cake and 920 U/g DM with coconut oil cake. Rodriquez *et al*., (2006) observed a similar efficacy for Triton X100 as the extractant for the lipase enzyme recovery. Mahadik *et al.,* (2002) also reported a similar trend when wheat

Fig.2. Screening of *C. gloesporioides* for extracellular lipase production by different seed oil cake

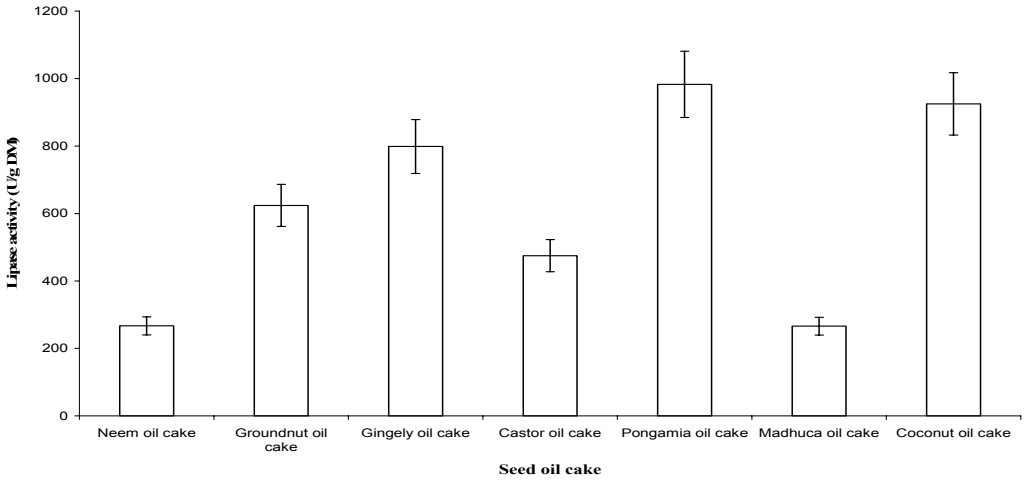


Fig.3. Effect of Moisture content on extracellular lipase production by *C. gloeosporioides* by solid state fermentation

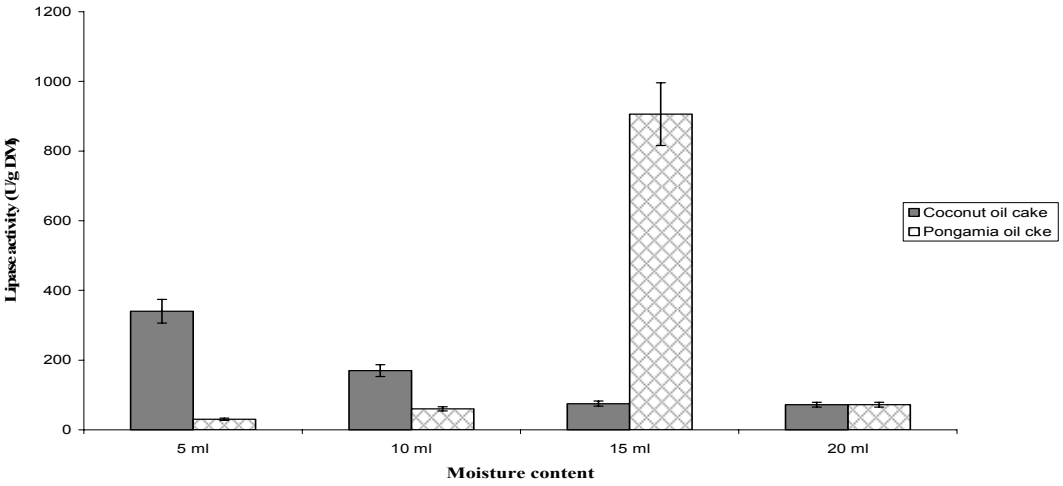


Fig.4. Extraction of extracellular lipases from C. gloeosporioides in solid fermented medium using different solutions

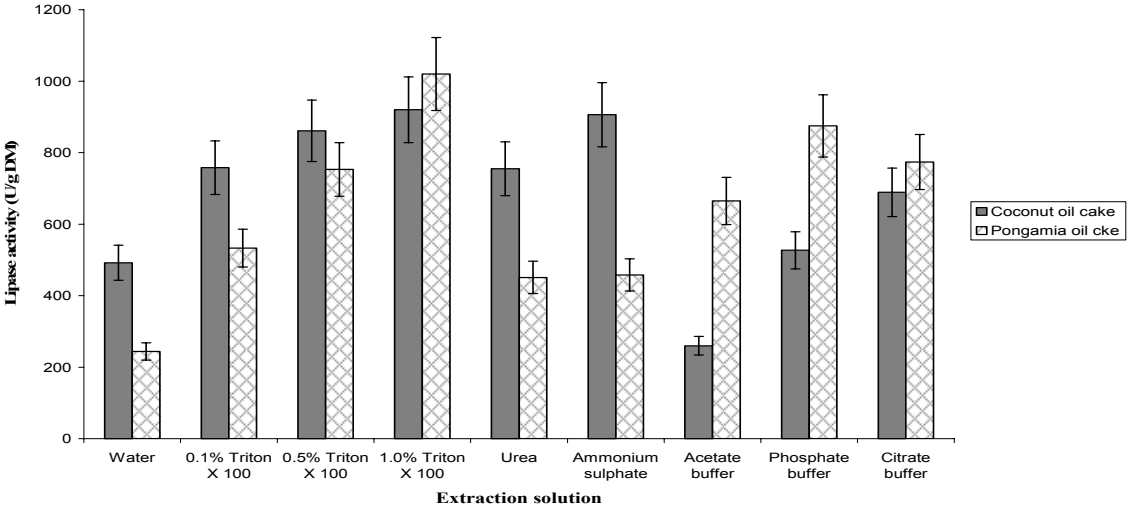


Fig.5. Effect of different temperature on extracellular lipase by solid state fermentation using *C. gloesporioides*

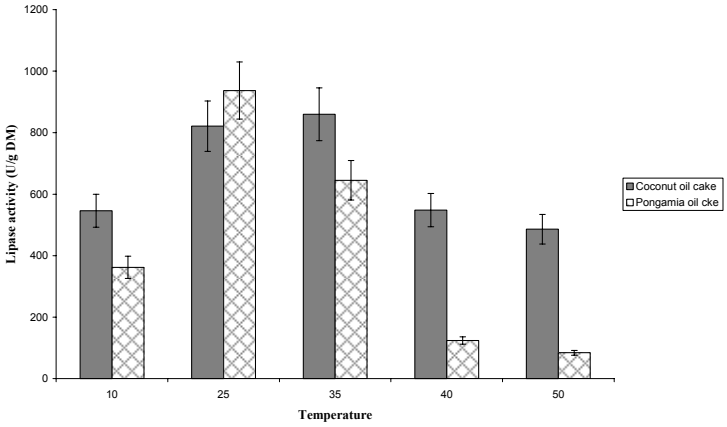


Fig.6.Effect of different carbon source on extracellular lipase by solid state fermentation using *C. gloeosporioides*

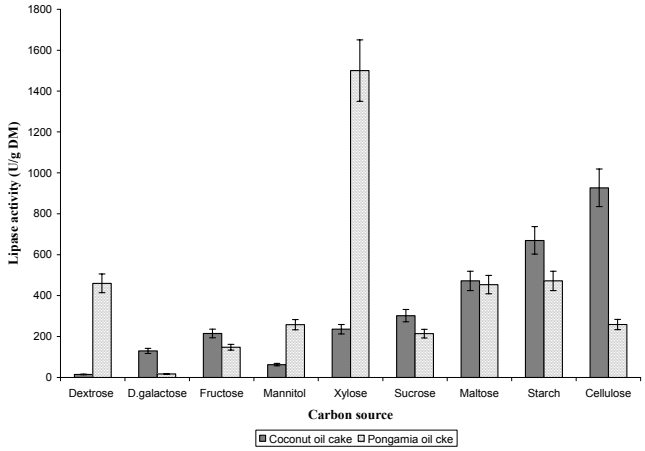
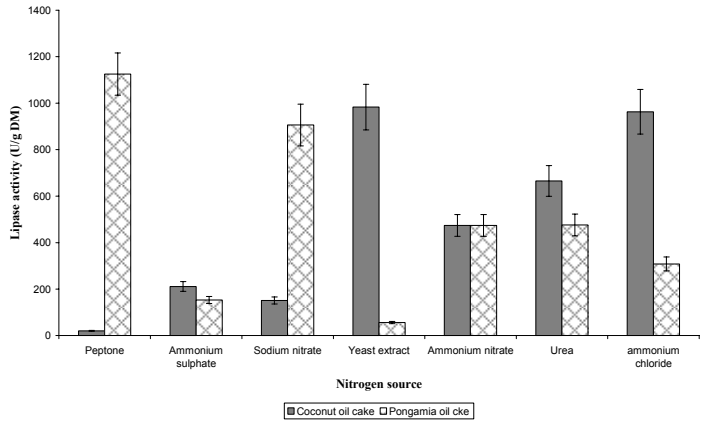


Fig.7. Effect of different nitrogen source on extracellular lipase by solid state fermentation using *C. gloeosporioides*



bran was extracted with sodium chloride (1%) supplemented with Triton X100. Other extractants such as ammonium sulphate and certain buffers did not match the efficacy of Triton X100. However, among the buffers tested Phosphate buffer proved as a good extractant (875 U/g DM). Perhaps this happens by the increased permeability as reported by Ul-Hag *et al*. (2002).

Temperature is another important environmental factor for enzyme synthesis. In this study, the optimum temperature varied with different substrates (Fig. 5). Accordingly, POC mediated enzyme activity was to the order of 937 U/g DM at 25^0 C while the same with COC yielding 860 U/g DM was at 35^0 C. Kamini *et al.* (1998) reported that for *A. niger* the temperature range was the same for enzyme secretion. As for the carbon and nitrogen sources for enzyme production, xylose with POC and peptone have been the best yielding 1500 U/g DM and 1125 U/g DM respectively (Fig. 6 & 7). But according to Rodriquez *et al.,* (2006) the nature of the carbon source does not have a pronounced effect. However, Tan *et al*., (2004) observed that peptone, an organic nitrogen source did yield higher enzyme in *P. camembertii*. According to Freire *et al*. (1997) peptone contains co-factors and amino acids which match *Pencillium* strains' physiological requirements.With respect to lipids as substrates or inducers for extracellular lipase production, Tween 60 provided the best lipid source even as sunflower oil appeared favourable inducer. Certain other substances such as Tween-20, Tween-40, cholesterol, triethanolamine, oleic acid, tristearin were not as good (Fig. 8 & 9) as Tween 60. The observation of Tan *et al*. (2004) reporting the suitability of the Tween series for best enzyme production holds true in our study as well. Tween-60 yielded 1170 U/g DM while olive oil yielded 2560 U/g DM with pongamia oil cake. Interestingly, with COC as the substrate, none of the lipid sources enhanced enzyme activity. Mahadik *et al.* (2002) reported that while working with *A. niger* on similar lines, SSF proved advantageous with

Fig.8. Effect of different Lipid source on extracellular lipase by solid state fermentation using *C. gloeosporioides*

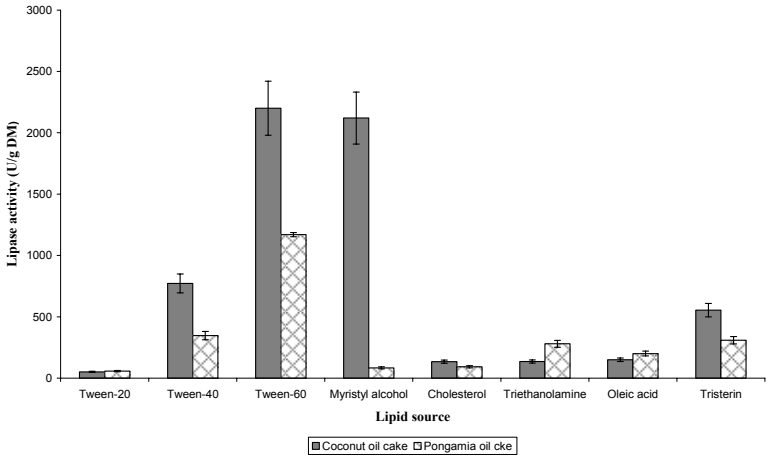


Fig.9.Effect of different Vegetable oil source on extracellular lipase by solid state fermentation using *C. gloeosporioides*

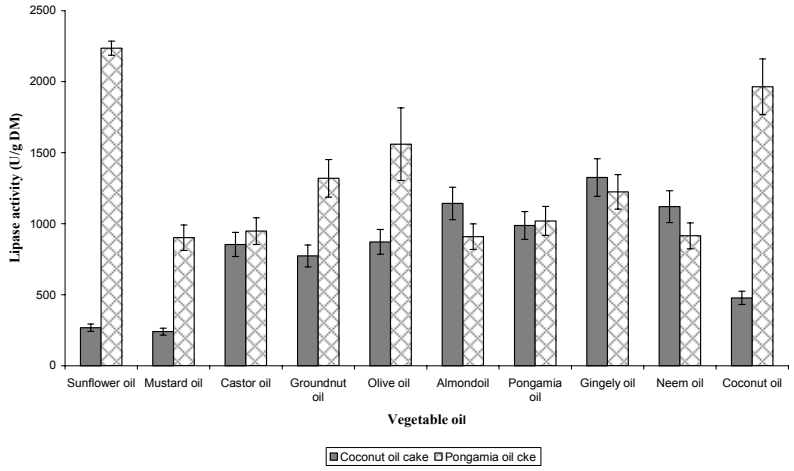
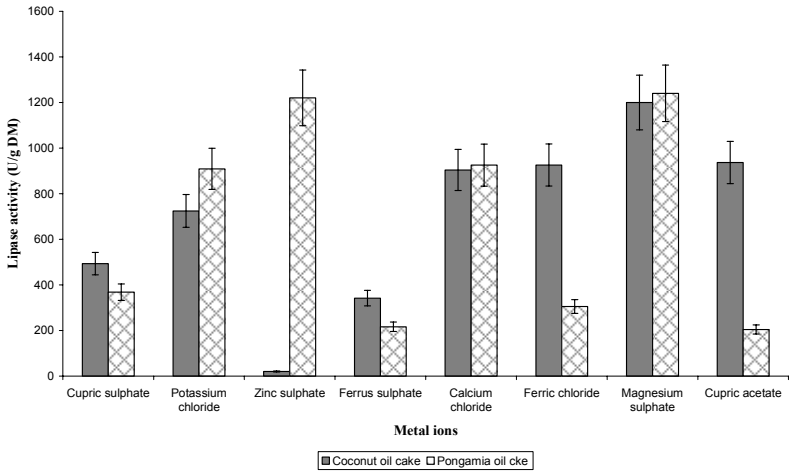


Fig.10. Effect of different metal ion source on extracellular lipase by solid state fermentation using *C. gloeosporioides*



wheat bran as the substrate and olive oil as both carbon and lipid sources for lipase secretion.

Metallic ions are believed to influence enzyme production and according to Tan *et al.,* (2003), magnesium (Mg^{2+}) sodium (Na^{+}) and potassium (K^{+}) are beneficial for the biosynthesis of lipases. In this study, Magnesium sulphate yielded 1240 U/g DM followed by Zinc sulphate 1220 U/g DM, calcium chloride 925 U/g DM, Potassium chloride 909 with POC as the substrate (Fig. 10). However, with COC, magnesium sulphate yielded 1200 U/g DM. Other metallic ions like Ca^{2+} are believed to form complexes with ionized fatty acids which alter their solubility and behavior at oil-water interfaces and thus inhibit the enzyme synthesis (Tan *et al*., (2004). However, Ca^{2+} does not appear to have inhibited lipase production in *C. gloeosporioides.*

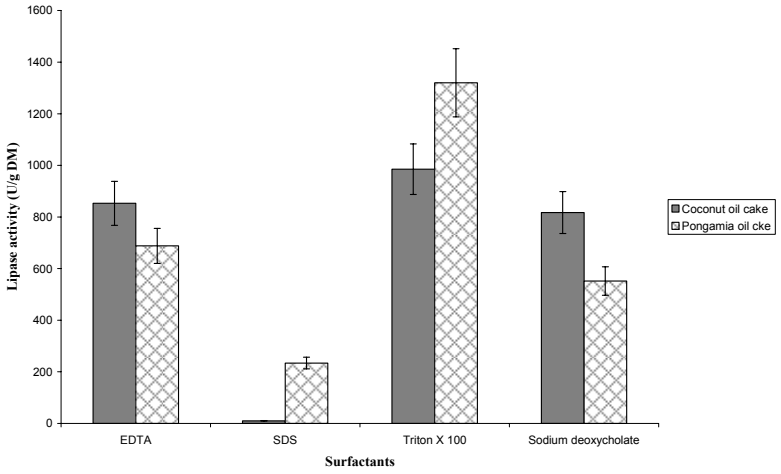
Besides providing an extractant, Triton X100 also functioned as the surfactant and increased lipase production was found in *C. gloeosporioides* (Fig. 11). Triton X 100 was also used as a surfactant earlier (Al-Asheh & Duvnjak,1994; Ebune *et al*.,1995) in SSF and observed increased enzyme activity under its influence.

Conclusions

Lipase production by endophytic fungi has not been adequately studied as revealed by the available literature. However, Coelomycetes constitute an interesting group of imperfect fungi with immense potential for biotechnology. In this investigation, extracellular lipase production by solid state fermentation appears to be promising in view of the cheap raw material that provided the fermentation substrates. Oil cakes, the left over residues are of little value except being used as cattle feed. But the same could be used for valuable product formation by fungi by way of solid state fermentation. This is encouraging because, cheap raw materials could

be explored for valuable enzyme production which has varied application in industry. This will cheer up the industrialist and therefore could be and for enzyme production on a commercial scale. However, further scaling-up studies are required.

Fig.11. Effect of Surfectants on extracellular lipase by solid state fermentation using *C. gloeosporioides*



References

1. Al- Asheh S and Duvnjak Z (1994) The effect of surfactants on the phytase production and the reduction of the phytic acid in canola meal by *Aspergillus carbonarius* using a solid state fermentation process. *Biotechnol. Lett*. 2, 183-188.
2. Benjamin S and Pandey A (1997) Coconut cake- a potent substrate for the production of lipase by *Candida rugosa* in solid-state fermentation. *Acta Biotechnol*. 17, 241-251.
3. Benjamin S and Pandey A (1998) Mixed-solid substrate fermentation. A novel process for enhanced lipase production by *Candida rugosa. Acta Biotechnol*. 18, 315-324.
4. Bogar B, Szak´acs G, Pandey A, Abdulhameed S, Linden JC and Tengerdy RP (2003) Production of phytase by *Mucor racemosus* in solid-state fermentation. *Biotechnol. Prog*. 19, 312-319.
5. Cammarota MC and Freire DMG (2006) A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. *Bioresour. Technol*. 97, 2195-2210.
6. Castilho LR, Polato CMS, Baruque EA, Sant'Anna jr GL and Freire DMG (2000) Economic analysis of lipase production by *Penicillium restrictum* in solid-state and submerged fermentations. *Biochem. Eng. J*. 4,239-247.
7. Colen G, Junqueira RG and Moraes- Santos T (2006) Isolation and Screening of alkaline lipase- producing fungi from Brazilian Savanna soil. *World J. Microbiol. Biotechnol*. 22, 881-885.
8. Cordova J, Nemmaoui M, Ismaili-Alaoui M, Morin A, Roussos S, Raimbault M and Benjilali B (1998) Lipase production by solid state fermentation of olive cake and sugar cane bagasse. *J. Mol. Catal. B: Enzym.* 5, 75-78.
9. Di Luccio M, Capra F, Ribeiro NP, Vargas GDLP, Freire DMG, Denise MG and De Oliveira D (2004) Effect of temperature, moisture, and carbon supplementation on lipase production by solid-state fermentation of soy cake by *Penicillium simplicissimum*. *Appl. Biochem. Biotechnol*. 113-116, 173-180.
10. Dominguez A, Costas M, Longo MA and Sanroman A (2003) A novel application of solid state culture: Production of lipases by *Yarrowia lipolytica*. *Biotechnol. Lett*. 25, 1225-1229.
11. Durand A (2003) Bioreactor designs for solid state fermentation. *Biochem. Eng. J*. 13,113-125.
12. Ebune A, Al- Asheh S and Duvnjak Z (1995) Effect of phosphates, surfactants and glucose on phytase production and hydrolysis of phytic acid in canola meal by *Aspergillus ficcum* during solid state fermentation. *Bioresour. Technol* . 54, 241-247.
13. Freire DMG, Teles EMF, Bon EPS and Sant Anna GL (1997) Lipase production by *Penicillium restrictum* in a bench-scale fermenter effect of carbon and nitrogen nutrition agitation and aeration. *Appl. Biochem. Biotechnol*. 63/64, 409-421.
14. Gombert AK, Pinto AL, Castilho LR and Freire DMG (1999) Lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate. *Proc. Biochem*. 35, 85-90.
15. Gutarra MLE, Godoy MG, Castilho LR, Freire DMG (2007) Inoculum strategies for *Penicillium simplicissimum* lipase production by solid-state fermentation using a residue from the babassu oil industry. *J. Chem. Technol. Biotechnol*. 82, 313-318.
16. H¨olker U, H¨ofer M and Lenz J (2004) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Appl. Microbial. Biotechnol*. 64, 175–186.

17. Kamini NR, Mala JGS, Puvanakrishnan P (1998) Lipase production from *Aspergillus niger* by solid-state fermentation using gingelly oil cake. *Proc. Biochem*. 33, 505-511.
18. Kwon DY and Rhee JS (1986) A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. *JAOCS* , 63 (1), 89- 92.
19. Leal MCMR, Cammarota MC, Freire DMG and Sant'Anna GL (2002) Hydrolytic enzymes as coadjutants in the anaerobic treatment of dairy wastewaters. *Brazil. J. Chem. Eng*.19, 175-180.
20. Macedo GA, Lozano MMS and Pastore GM (2003) Enzymatic synthesis of short chain citronellyl esters by a new lipase from *Rhizopus* sp. *Electr. J. Biotechnol*. 6, (http://www.ejbiotechnology.info/content/ vol6/issue1/full/2/2.pdf).
21. Mahadik ND, Puntambekar US, Bastawde KB, Khire JM and Gokhale DV (2002) Production of acidic lipase by *Aspergillus niger* in solid state fermentation. *Proc. Biochem*. 38 (5), 715-721.
22. Olama ZA and El-Sabaeny AH (1993) Lipase production by *Aspergillus niger* under various growth conditions using solid state fermentation. *Microbiologia (Madrid)* 9, 134-141.
23. Ortiz-Vazquez E, Granados-Baeza M and Rivera-Munoz G (1993) Effect of culture conditions on lipolytic enzyme production by *Penicillium candidum* in a solid state fermentation. *Biotechnol. Adv*. 11, 409-416.
24. Palma MB, Pinto AL, Gombert AK, Seitz KH, Kivatinitz SC, Castilho LR and Freire DMG (2000) Lipase production by *Penicillium restrictum* using solid waste of industrial Babassu oil production as substrate. *Appl. Biochem. Biotechnol*. 84-86, 1137- 1146.
25. Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N and Soccol VT (1999a) The realm of microbial lipases in biotechnology. *Biotechnol. Appl. Biochem*. 29, 119-131.
26. Pandey A, Selvakumar P, Soccol CR and Nigam P (1999b) Solid state fermentation for the production of industrial enzymes. *Curr. Sci*. 77, 149-162.
27. Pandey A, Soccol CR and Mitchell D (2000) New developments in solid state fermentation: 1-bioprocesses and products. *Proc. Biochem*. 35, 1153-1169.
28. Raimbault M and Alazard D (1980) Culture method to study fungal growth in solid fermentation. *Eur. J. Appl. Microbiol. Biotechnol.* 9, 199-209.
29. Ramachandran S, Patel AK, Nampoothiri KM, Franics F, Nagy V and Szakacs G (2004) Coconut oil cake – a potential raw material for the production of α – amylase. *Bioresour. Technol*, 93, 169-174.
30. Rao PV, Jayaraman K and Lakshmanan CM (1993a) Production of lipase by *Candida rugosa* in solid state fermentation. 1: determination of significant process variables. *Proc. Biochem*. 28, 385-389.
31. Rao PV, Jayaraman K and Lakshmanan CM (1993b) Production of lipase by *Candida rugosa* in solid state fermentation. 2. Medium optimization and effect of aeration *Proc. Biochem*. 28, 391-395.
32. Rivera-Munoz G, Tinoco-Valencia JR, Sanchez S and Farres A (1991) Production of microbial lipases in a solid state fermentation system. *Biotechnol. Lett*. 13, 277-280.
33. Rodriguez JA, Mateos JC, Nungaray J, Gongalez V, Bhagnagar T, Roussos S, Cordova J and Baratti J (2006) Improving lipase production by nutrient source modification using *Rhizopus homothallicus* cultured in solid state fermentation. *Proc. Biochem*. 41, 2264-2269.
34. Sharma R, Chisti Y and Banerjee UC (2001) Production, purification, characterization, and applications of lipases. *Biotechnol*.19, 627-662.
35. Sugiura M (1984) Bacterial lipases. In: Borgstrom B, Brockman H.L, editors. Lipases. Amsterdam: Elsevier. 505-523.
36. Sumitra R, Singh SK, Larroche C, Soccol CR and Pandey A (2007) Oil cakes and their biotechnological applications- A review. *Bioresour. Technol.* 98 (10), 2000- 2009.
37. Tan T, Zhang M, Wang B, Ying C and Deng L (2003) Screening of high lipase production *Candida* sp. and production of lipase by fermentation. *Proc. Biochem*. 39, 459-465.
38. Tan T, Zhang M, Xu J and Zhang J (2004) Optimization of culture conditions and properties of lipase from Penicillium camembertii Thom PG-3. *Proc. Biochem*. 39,1495-1502.
39. Ul-Haq I, Idrees S and Rajoka MI (2002) Production of lipases by *Rhizopus oligosporous* by solid-state fermentation. *Proc. Biochem*. 37, 637-641.
40. Uvarani G, Jaganathan L, Shridas P and Boopathy R (1998) Purification and characterization of lipase from *Rhizomucor miehei*. *J. Sci. Ind. Res*. 57, 607-610.