ISSN: 0975-766X
CODEN: IJPTFI
Available Online through
www.ijptonline.com
Research Article

SUBMERGED FERMENTATION OF *ASPERGILLUS NIGER* LIPASE PRODUCTION BY USING TANNERY SOLID WASTE

A. Manikandan, N. R. Kamini, P.B. Ramesh Babu
Department of Bioinformatics, Bharath University, Chennai, Tamilnadu, India.
Department of Biotechnology, Central leather research Institute, Chennai, India.
Department of Bioinformatics, Bharath University, Chennai, Tamilnadu, India.

Received on 14-07-2015 *Accepted on 30-07-2015*

Abstract

Beef fleshing has been used as a nutrition source for the production of lipase from *Aspergillus niger*. Cost effect medium components, namely, CSL, $NH_4H_2PO_4$, Na_2HPO4, and olive oil, which were found to be influential for lipase production. Optimum values of the influential parameters were CSL, 2.0%, w/v; NH4H2PO4, 0.05%, w/v; Na2HPO4, 0.75%, w/v; and olive oil, 2.0%, w/v, had a significant impact on lipase production with an activity of 25.8 U/mL at 48 h and 30 °C, which was higher than the initial activity (20 U/mL). Furthermore, the enzyme has good potential for the hydrolysis of beef fleshing and a hydrolytic ratio of 91.67% was obtained at 72 h. Enhanced production of lipase by using beef fleshing – 2%. The utilization of CSL and beef fleshing for lipase production from *A. niger* makes the process green, because both are renewable substrates and economically viable at an industrial scale.

Introduction

Corn steep liquor (CSL), a byproduct of the corn wet-milling industry, is used as an ingredient in animal feed and as a nutrient supplement for microorganisms in diverse industrial fermentation processes. It is a mixture consisting of water-soluble extracts of corn soaked (steeped) in water, composed entirely of natural amino acids, minerals, vitamins, reducing sugars, organic acids, enzymes, and other elemental nutrients, which are excellent source of nutrients for microorganisms (1,2). Themost important application of CSL in microbiology was initially discovered by Moyer and Coghill (3), who noticed that the addition of CSL to the liquid medium greatly increased the yields of penicillin from Penicilliumnotatum. Later,Gernet al. (4), Lotfy et al. (5), Essamri et al. (6), and Schroeder (7) reported that supplementation of CSL to the growthmediumincreased themycelial growth of Pleurotus ostreatus, Aspergillus

niger, Rhizopus oryzae, and Aspergillus parasiticus, respectively, because CSL also adjusts the tracemetal balance in the fermentation medium.

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, 1999). Lipase 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., 1999; 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)

Material and Methods

Material

All the chemicals used in the present study were of AR grade and purchased from Hi-Media Limited and S.D. Fine Chemicals Limited, Mumbai, India. Corn steep liquor was obtained as gifts from Anil Products Limited, Mumbai,

India. The other ingredients of the media, namely, sucrose, sodium chloride, and olive oil, were purchased from local markets in Chennai, India.

Inoculum and Culture Conditions

The spore suspension for inoculation was prepared by adding 2 ml of sterile distilled water to the culture slant, and the spores were dislodged using an inoculation needle under aseptic conditions[1]. A spore suspension containing 4.3 × 10^8 spores/ ml was used as an inoculum. Lipase production was carried out in 250mL Erlenmeyer flasks, each containing 10 ml of the sterile production medium of pH 7.0. The medium was inoculated with 240 µl of spore suspension and grown for 48 h on a rotary shaker at 30 °C and 120 rpm. After growth, the biomass was removed by filtration, and the cell-free supernatant was used as crude enzyme preparation. All of the experiments were carried out in batches, and the results are expressed as the average of triplicates[2].

Estimation of Protein Content (Kjeldhal method or Digestion, 1883)

20ml of concentrated H_2SO_4 with catalyst (Potassium sulphate and copper sulphate) to one gram of beef flesh. Addition of 50ml distilled water and further 50ml of baric acid added. Finally back titrated with 0.03N concentrated sulphuric acid. Khjeldhal method is used to estimate the nitrogen content of sample[3]. The conversion factor (6.25) can be used to predict the quantity of protein present in sample.

$$\text{Nitrogent (\%)} = \frac{(A-B) \times \text{Normality of } H_2SO_4 \times \text{Mw of Nitrogen}}{\text{Weight of the sample}} \times 1000$$

Extraction Lipids from Beef Fleshing using Soxhlet Method: 12g of Sample (Beef fleshing) with 150ml of petroleum ether. Sample was reflux for about 4- 5 hours and solvent evaporated in boiling water bath. Weigh the lipid content in sample[4].

Moisture Analysis of Beef Fleshing: 1g of sample was incubated at 100 °C in hot air oven for 1hour and Sample weighs in moisture analyzer

Ash Content of Beef Fleshing: Fleshing maintained in 500 - 600 °C for 1 hour and fleshing became ash, sample weighed in the balance.

Medium Optimization with CSL as Nutrition Adjunct: Lipase production was initially carried out in the production medium of Kamini et al. (26) with the following composition (%, w/v):meat extract, 1; urea, 0.5; sucrose, 0.5; MgSO4 3 7H2O, 0.05; KH2PO4, 0.1; Na2HPO4, 0.3; and olive oil, 1. To develop a low-cost medium,

optimization studies were investigated using by CSL (0.5-5.0%, w/v), urea, sucrose, sodium chloride, various inorganic nitrogen sources [NH4NO3, (NH4)2SO4, NH4H2PO4, and NaNO3], phosphates (KH2PO4 and Na2HPO4), and inducers (olive oil) were replaced by beef fleshing for the production of lipase[5].

Enzyme Assay: Lipase activity was determined according to the method of Yamada et al. (1965) using olive oil as substrate[6]. One unit of activity was defined as the amount of enzyme releasing 1 μmol of free fatty acid in 1 min at pH 7.0 and 37°C

Replacement of Oil Inducers

Beef fleshing used to replace the various oil as inducers and increase the production of lipase enzyme by submerged fermentation[6]. The beef fleshing about 2% was used in the production medium

Scale up of Lipase Production

Scales up of lipase production from 10ml flask level to 200ml were carried out. Substrate inoculums were inoculated into 200ml of CSL medium in series of sterilized flasks 96 h and assayed for lipase activity at 24 h intervals. The variation of temperature during the process was monitored[7].

Hydrolysis of Various Oils by *A. niger* Lipase

Crude culture filtrate containing lipase of A. niger was subjected to ammonium sulfate precipitation (80% saturation)[8]. The resulting precipitate was dissolved in 0.01 M phosphate buffer (pH 7.0) and dialyzed against the same buffer. The partially purified lipase was lyophilized and used for the hydrolysis of vegetable oils and fish oils[9].

Hydrolytic reactions were carried out in series of 100 mL Erlenmeyer flasks containing 1 g of oil, 15mLof 0.1Mphosphate buffer (pH 7.0), and 50 U of lipase (81.74 U/mg of protein) and incubated at 30 °C for 120 h with shaking at 120 rpm. Samples were taken at 24 h intervals, and reaction was stopped by the addition of 20 mL of ethanol; the free fatty acids liberated were titrated with 0.1 N KOH[10]. A control was carried out similarly, except the enzyme solution was added after the addition of ethanol. The control value was subtracted from the experimental value, and the acid value was calculated. The hydrolysis ratio was calculated by the following equation[12]:

Hydrolysis ratio (%) = acid value/saponification value ×100

The effects of lipase concentration, substrate/buffer ratio, and use of additives and solvents in the reaction mixture were also determined for palm oil to achieve maximum hydrolysis[13]. The fatty acid composition of the hydrolyzed

palm oil was estimated by esterification of the fatty acids to their respective methyl esters as described by Kanya et al. (28).

Results and Discussion

Beef Fleshing Characterization

Proximate analysis of crude protein, fat, ash and moisture were carried out according to the methods of AOAC[14]. All determinations were done in triplicate and the mean value was recorded. Protein content was higher than lipid content noted in all tested samples[15].

Fig 1: Composition of Beef Fleshing.

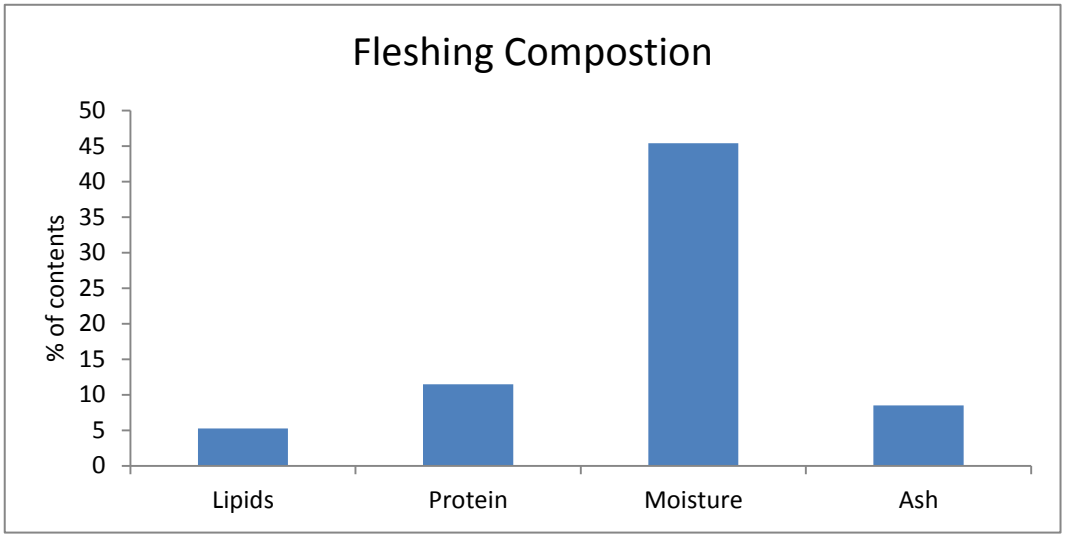


Table 1: Composition of Beef Fleshing.

S.No	Composition	Percentage
1	Lipids	5.27
2	Protein	11.5
3	Moisture	45.4
4	Ash	8.5

Submerged Fermentation

The production of lipase from *A. niger* in submerged fermentation was reported earlier by Kamini *et a[17]l.* 1998. Economic analysis of the above medium revealed that addition of meat extract alone to the medium contributes about 66.8% of medium cost. Therefore, attempts were made to make the process economically viable by using an inexpensive nutrition adjunct, CSL, because it is an excellent source of nitrogen and carbon for most of the microorganisms[18].

Fig 2: Lipase Production by Conventional Medium

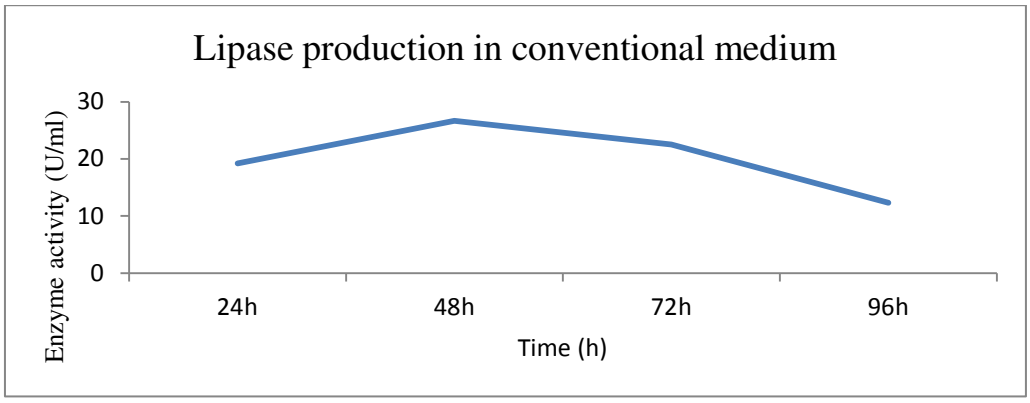
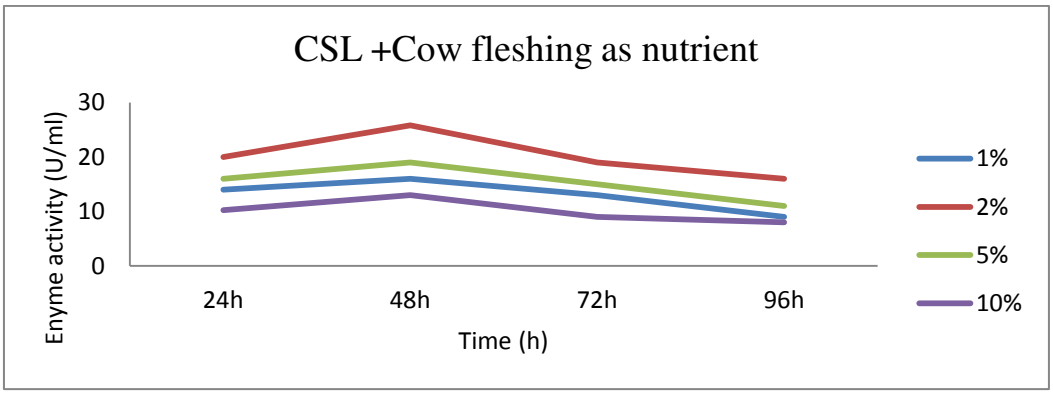


Table 2: Lipase Production using CSL and Oil as Inducers.

Time (h)	Activity (U/ml)
24h	19.2
48h	25.5
72h	22.5
96h	12.3

Conventional medium has got higher yield of 25.5 (U/ml) at 48h. Oil inducer plays vital role in production medium along with other ingredient[19].

Fig 3: Lipase Production using Beef Fleshing and CSL.



Addition of beef fleshing for lipase production in submerged fermentation show comparable enzyme production (25.8 U/ml) at 48h.Exctraction process made easy when we using the beef fleshing in production medium[20].

Table 3: Lipase Production using Beef Fleshing and CSL.

Percentage of Fleshing	24h	48h	72h	96h
1%	14	16	13	9
2%	20	25.8	19	16
5%	16	19	15	11
10%	10.2	13	9	8

Beef fleshing which replace the oil inducer in lipase production medium at different ratio (1%, 2%, 5%, 10%) among the different ratio the 2% of beef fleshing had influence on the lipase production[21]

Hydrolysis of Beef Fleshing using Lipase from *Aspergillus niger*

The fleshing was hydrolyzed efficiently to 71.3% at 24 h initially and optimization studies showed an increase in the hydrolytic ratio by 15.6% at 24 h, when the reaction mixture contained 10 g of fleshing, 25 ml of 0.1 M phosphate buffer (pH 7.0), and 250 U of lipase[22]. Accordingly, the fermented substrate could be directly used for the hydrolysis of tallow and in the production of fatty acid esters, since the fuel produced from tallow has the advantage of a higher calorific value and octane number than the fuels obtained from vegetable oils as reported by Lebedevas *et al*. (2006)[23]. Moreover, the reported hydrolytic ratio was comparatively higher with our lipase, than the hydrolytic ratios of beef fleshing obtained with lipases from Rhizomucor miehei (73%) and Yarrowia lipolytica (65%) (Adamczak and Bednarski, 2004), while a higher hydrolytic ratio of 93.86% was reported by Gao *et al*. (2009) using surfactant coated commercial lipase of C. Rugosa (Novoenzymes, China). However, the direct application of the fermented substrate for fleshing hydrolysis by *A[24]. niger* makes the process economical and avoids the need for expensive enzyme recuperation and immobilization processes[25].

Fig 4: Hydrolysis of Fleshing by *A. niger* Lipase.

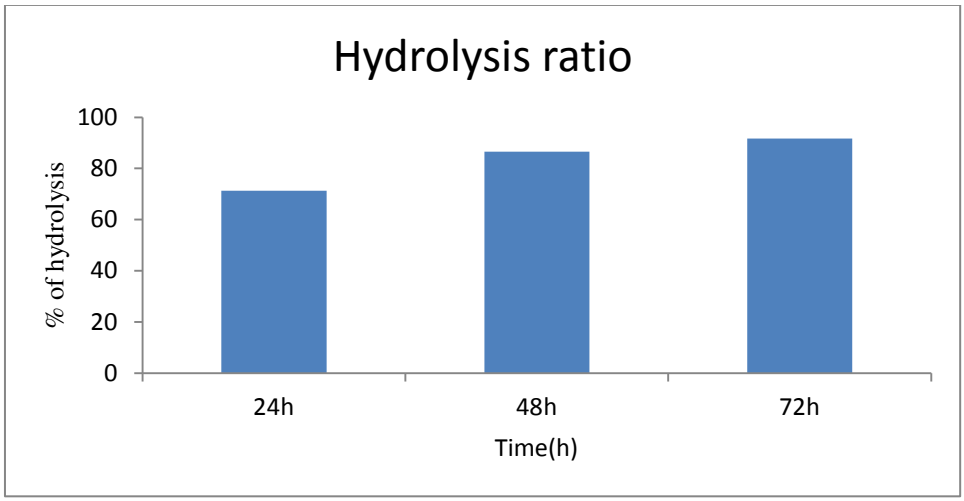


Table 4: Percentage of Fleshing Hydrolysis by Lipase.

S.No	Incubation	Hydrolysis Percentage
1	24h	71.30
2	48h	86.58
3	72h	91.67

The direct application of fermented substrate on fleshing hydrolysis was evaluated, since the use of fleshing is declined due to the changing feeding habits of human beings and all the excess tallow produced are not used in soap industry (Bhatti *et al*., 2008). Hence, it is economical to consider fleshing, a low cost feedstock, in oleochemical industries for the production of fatty acids and their corresponding esters using enzymes, because they make the process energy efficient than the conventional thermal[26] fat splitting process, which requires operations at elevated temperature and pressure (Edwinoliver *et al*., 2010).

References

1. Adamczak, M. and Bednarski, W. "Enhanced activity of intracellular lipases from *Rhizomucor miehei* and *Yarrowia lipolytica* by immobilization on biomass support particles", Process Biochem., Vol. 39, pp.1347–1361, 2004.
2. Adinarayana, K., Prabhakar, T., Srinivasulu, V., Rao, M.A., Lakshmi, P.J. and Ellaiah, P. "Optimization of process parameters for cephalosporin C production under solid state fermentation from *Acremonium chrysogenum*". Process Biochem., Vol. 39, 171–177, 2003b.
3. Babu, I.S. and Rao, G.H. "Lipase production by *Yarrowia lipolytica* NCIM 3589 in solid state fermentation using mixed substrate", Res. J. Microbiol. Vol. 2, pp.469–474, 2007.
4. Benjamin, S., Pandey, A., "Coconut cake: a potent substrate for production of lipase by *Candida rugosa* in solid state fermentation", Acta Biotechnol., Vol. 17, pp.241–251, 1997.
5. Bhatti, H.N., Hanif, M.A., Qasim, M. and Rehman, A. "Biodiesel production from waste tallow", Fuel, Vol. 87, pp.2961–2966, 2008.
6. Cordova, J., Nemmaoui, M., Ismaili-Alaoui, M., Morin, A., Roussos, S., Raimbault, M. and Benjilali, B. "Lipase production by solid state fermentation of olive cake and sugarcane bagasse". J. Mol. Catal. B: Enzym., Vol. 5, pp.75–78, 1998.
7. Edwinoliver N.G., Thirunavukarasu K., Naidu R.B., Gowthaman M.K., Nakajima Kambe T., Kamini N.R. "Scale up of a novel tri-substrate fermentation for enhanced production of *Aspergillus niger* lipase for tallow hydrolysis", Bioresour. Technol., Vol. 101, pp. 6791–6796, 2010.
8. Hasan, F., Shah, A.A. and Hameed A. "Industrial applications of microbial lipases'. Enzy. Microbial. Technol., Vol. 39, pp. 235-251, 2006.

9. Kamini, N.R., Mala, J.G.S., Puvanakrishnan, R. "Lipase production from *Aspergillus niger* by solid state fermentation using gingelly oil cake". Process Biochem., Vol. 33, pp. 505–511, 1998.
10. Kanya, T. C. S.; Rao, L. J.; Sastry, M. C. S. Characterization of wax esters, free fatty alcohols and free fatty acids of crude wax from sunflower seed oil refineries. Food Chem. 2007, 101, 1552–1557.
11. Lebedevas, S., Vaicekauskas, A., Lebedeva, G., Makareviciene, V., Janulis, P., Kazancev, K. "Use of waste fats of animal and vegetable origin for the production of biodiesel fuel: quality, motor properties, and emissions of harmful components", Ener. Fuel, Vol. 20, pp. 2274–2280, 2006.
12. Mahadik, N.D., Puntambekar, U.S., Bastawde, K.B., Khire, J.M., Gokhale, D.V. "Production of acidic lipase by *Aspergillus niger* in solid state fermentation", Process Biochem., Vol.38, pp. 715–721, 2002.
13. Mahanta, N., Gupta, A., Khare, S.K. "Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* PseA in solid state fermentation using Jatropha curcas seed cake as substrate",. Bioresour. Technol. Vol. 99, pp.1729–1735, , 2008.
14. Mala, J.G.S., Edwinoliver, N.G., Kamini, N.R., Puvanakrishnan, R. "Mixed substrate solid state fermentation for production and extraction of lipase from *Aspergillus niger* MTCC 2594", J. Gen. Appl. Microbiol., Vol. 53, pp. 247–253, , 2007.
15. Rao, P., and Divakar, S. "Lipase catalyzed esterification of a-terpineol with various organic acids: application of the Plackett-Bunnan design", Process Biochem., vol. 36, pp.1125-1128, 2001.
16. Rodriguez, J.A., Mateos, J.C., Nungaray, J., Gonzalez, V., Bhagnagar, T., Roussos, S., Cordova, J., Baratti, J. "Improving lipase production by nutrient source modification using *Rhizopus homothallicus* cultured in solid state fermentation", Process Biochem., Vol.41, pp. 2264–2269, 2006.
17. Tschocke, C. "Enzymatic treatment of fats in wastewater treatment plants", Eau. Ind. Nuisances, Vol.138, pp.63-64, 1990.
18. Yamada, K.; Ota, U.; Machida, H. Studies on the production of lipase by microorganisms II. Quantitative determination of lipase. Agric. Biol. Chem. 1962, 26, A69.
19. Niranjan U., Subramanyam R.B.V., Khanaa V., "Developing a Web Recommendation System Based on Closed Sequential Patterns", Communications in Computer and Information Science, ISSN : 1865-0929, 101() (2010) PP.171-179.

20. Anbuselvi S., Jeyanthi Rebecca L., Sathish Kumar M., Senthilvelan T., "GC-MS study of phytochemicals in black gram using two different organic manures", Journal of Chemical and Pharmaceutical Research, ISSN : 0975 – 7384, 4(2) (2012) PP. 1246-1250.
21. Lydia Caroline M., Vasudevan S.,"Growth and characterization of l-phenylalanine nitric acid, a new organic nonlinear optical material", Materials Letters, ISSN : 0167-577X, 63(1) (2009) pp. 41-44.
22. Langeswaran K., Gowthamkumar S., Vijayaprakash S., Revathy R., Balasubramanian M.P., "Influence of limonin on Wnt signalling molecule in HepG2 cell lines", Journal of Natural Science, Biology and Medicine, ISSN : 0976-9668, 4(1) (2013) PP. 126-133.
23. Jayalakshmi T., Krishnamoorthy P., Ramesh Kumar G., Sivamani P., "Optimization of culture conditions for keratinase production in Streptomyces sp. JRS19 for chick feather wastes degradation", Journal of Chemical and Pharmaceutical Research, ISSN : 0975 – 7384, 3(4) (2011) PP.498-503.

25.Thirunavukarasu, K.; Edwinoliver, N. G.; Duraianbarasan, S.; Gowthaman, M. K.; Iefuji, H.; Kamini, N. R. Removal of triglyceride soil from fabrics by a novel lipase from Cryptococcus sp S-2. Process Biochem. 2008, 43, 701–706.

26.Yamada, K.; Ota, U.; Machida, H. Studies on the production of lipase by microorganisms II. Quantitative determination of lipase. Agric. Biol. Chem. 1962, 26, A69.

Corresponding Author:

A. Manikandan*,

Bharath University, Chennai, Tamilnadu, India.