International Research Journal of Engineering and Technology (IRJET) e-ISSN: 2395-0056
Volume: 02 Issue: 09 | Dec-2015 www.irjet.net p-ISSN: 2395-0072

Synthesis of food ester butyl acetate by immobilized *Aspergillus oryzae* (NCIM 1212)lipase

Dabeer S.[1], Satpute V[1]. , Shendre R[1]., Trigune H[1] and Mane A[2]

[1] *Department of Biotechnology, KIT's College of Engineering, Kolhapur, India*

[2]*Department of Chemistry, KIT's College of Engineering, Kolhapur, India*

Abstract- *The production of lipase was carried out by A. oryzae (NCIM 1212) by solid state fermentation using agriculture wastes. An enzyme activity of 6.248 U/ml/min was obtained by pNPP assay. Pineapple flavourd butyl acetate was synthesized from acetic acid and butanol in a solvent free system with the immobilized lipase enzyme in the form of CLEAs. Several parameters in the esterification reaction were optimized. The maximum % conversion of butyl acetate (80%) was obtained at 37⁰ C in 72 hours with the enzyme dose of 10% w/w; molar ratio of acetic acid to butanol, 1:0.5 in esterification reaction. Significant synthesis activity was observed for first three cycles of reuse of immobilized lipase. The product was confirmed as butyl acetate from FTIR analysis whereby the presence of an ester group was observed at wave number of 1721 cm⁻¹.The use of solid state fermentation and immobilized lipases, both have increased the economy in this green synthesis of food esters.*

Keywords- butyl acetate, CLEAs, esterification, lipase, solid state fermentation (SSF)

1. Introduction- Flavour esters are compounds of a great commercial importance due their application mainly in food, cosmetic and pharmaceutical industries [1, 13].

Traditional chemical synthesis or their extraction from plant materials are costly and also low yielding making these techniques inadequate at large scale.[1, 6] On the other hand, the main advantages of the lipase catalyzed flavour generation are high selectivity, high reaction rate even at mild reaction conditions and the process is completely green [1,13,14]. Butyl acetate, a flavor ester can be successfully synthesized using lipase as biocatalyst in the esterification reaction between acetic acid and butanol. [13]

Lipase can be produced either by submerged fermentation[4]or by solid state fermentation (SSF)[6, 15] .In SSF, the use of cheap raw materials would diminish the operating costs of the process. Moreover, cost for lipase production has been reported to be significantly lower than in submerged fermentation. Due to superior productivity, reduced energy requirements, low wastewater output, improved product recovery etc. [9, 12].

In this study, we have produced lipase by solid state fermentation using linseed oil cake. Furthermore, the crude lipase produced is immobilized by using CLEA technique and it has been used in the synthesis of short chain food ester, butyl ester by direct esterification reaction in solvent-free system. Different reaction parameters were investigated to achieve maximum conversion yield.

2. Materials and methods:

2.1 Chemicals:

All the chemicals used in the present study were of analytical grade and procured from Himedia, Ranchem, Qualigens, Sigma-aldrich, Bangalore Genei etc.

2.2 Microorganism:

Aspergillus oryzae (NCIM No. 1212) with lipolytic activity[3]was procured from National Collection of Industrial Microorganism (NCIM), Pune (India). The cultures were reviewed & stored on maintenance media and renewed regularly after three weeks throughout the study.

2.3 SSF Based Production Studies:

The SSF based studies on production of lipase was done considering parameters like screening of the substrate, moisturizing agent and preparation of inoculum. Different agro wastes were screened as a substrate such as oil cakes of cotton seed, linseed, and local varieties like kardai, and javas. Nutrient broth with composition (peptone 10 g/l, sodium chloride 5 g/l, yeast extract 5 g/l) was used as moisturizing agent. 5 g of substrates were suspended in 15 ml of nutrient broth media in 250 ml flasks after sterilization (15 lbs, 121^{0}C for 20 min). The Fermentation was carried out at 27^{o}C for 24 hours with 5 ml of inoculum. The enzyme extract was pooled after centrifugation of the fermented matter after washing with10 ml of tris-HCl buffer (pH-8) and squeezing through a wet muslin cloth. The substrate giving maximum lipase activity was used for optimization study and large scale production. Lipase activity was determined by pNPP assay as described by Gupta et al.[20]

2.4 Immobilization of Lipase:

Crude lipase was immobilized as Cross linked enzyme aggregates (CLEAs) using 70% (w/v) ammonium sulphate and 2% (v/v) glutaraldehyde as described by Wilson et al. [16,18]

2.5 Esterification reaction:

Reactions were carried out in 100 ml screw capped flasks containing various acid (acetic acid) to alcohol (butanol) molar ratios and different amounts of CLEAs. The flasks were incubated at different temperatures on rotary shaker with speed 150 rpm for different time intervals.

2.6 Analysis of reaction product:

Aliquots of reaction mixture were withdrawn periodically**.** The yield of butyl acetate was determined by titration method with 0.1 N NaOH and was expressed as a percent (%) of converted acetic acid as compared to the total acid in the reaction mixture or relative percentage conversion (%).

2.7 Identification of reaction product:

The synthesized butyl acetate was analyzed using Fourier transform infrared spectroscopy (FTIR).It was also compared with chemically synthesized butyl acetate using thin layer chromatography. Hexane and diethyl ether were used in the developing solvent system in ratio of 9:1 (v/v). For chemical synthesis of butyl acetate, the reaction mixture containing 0.05M acetic acid and 0.06M butanol was heated at 80^{0}C for 10 hours on magnetic rotar with H_2SO_4 as a catalyst.

2.8 Reusability:

The enzyme was recovered after each cycle by centrifugation at 10000 rpm for the next esterification reaction and the % conversion yield was calculated after each cycle.

3. Results and Discussion: (Values given in the results are means of three independent experiments.)

3.1 SSF based production of lipase: As the maximum lipase activity (4.562 U/ml.min) was

found with linseed cake in the initial screening, the same was used as a substrate for large scale solid state fermentation. The activity of produced lipase was determined by pNPP assay. The activity of lipase produced in large scale production was slightly greater than that obtained in pilot studies (6.2489 U/ml/min), indicating the efficiency of process. Immobilization by CLEA preparation was successfully carried out. Activity recovery of lipase after immobilization was found to be 71.04 %. Little less activity recovery may be due to less lysine residues on the surface of lipase. It may be increased by co aggregation technique. [18]

3.2 Effect of acid to alcohol molar ratio:

The effect of acetic acid to butanol molar ratio was investigated using immobilized *A. oryzae* lipase in a solvent-free system. Butyl acetate production was found to be maximum at the ratio of 1:0.5 (table1) which means there was maximum conversion of acetic acid into an ester (80%). The % yield decreased drastically when solvent concentration was increased to 1:1 ,1:2 which was nearly half (42.85%) and it was least (20%) at 1:3 which may be probably due to lipase denaturation by acetic acid.Hence, solvent ratio of 1:0.5 was used for further optimization studies.

Table 1 Effect of acid to alcohol molar ratio on esterification

Reaction conditions: Time: 72 hours, temperature: 37^{0}C, agitation: 150rpm, Enzyme concentration 10% w/w

Ratio of Acetic acid : Butanol	% conversion
1: 0.5	80
1: 1	42.85
1: 2	42.85
1: 3	20

.

3.3. Effect of reaction time on esterification reaction:

Time course studies help to determine the shortest time necessary for the cost-effectiveness of the process. Generally, the relative percentage conversion of butyl acetate was increased with increasing reaction time. The % conversion at different time internals is shown in Table 2.

Table 2 Effect of reaction time on esterification
Reaction conditions: Temperature: 37^{0}C, agitation: 150rpm, acetic acid: butanol ratio1:0.5, enzyme concentration: 10% w/w

Time in hours	% conversion
6	0
18	0
24	13.33
42	33.33
48	40
66	73.33
72	73.33

3.4. Effect of temperature on esterification reaction:

The relative percentage conversion of ester was maximum at temperature 37°C.However, at higher temperatures, the relative percentage of yield was gradually decreased from 45-50°C. This is probably due to the thermal deactivation of lipase.

Table 3 Effect of temperature time on esterification Reaction conditions: Time: 72 hrs, agitation: 150rpm, acetic acid: butanol ratio1:0.5, enzyme concentration: 10% w/w

Temperature(^{0}C)	% conversion
37	80
45	46.66
50	13.33

3.5 Effect of amount of enzyme on esterification reaction:

High substrate concentration with low enzyme concentration in the esterification reaction is desirable taking in consideration an industrial application of the reaction. [13] The % conversion at different enzyme concentration is shown in table4.

Table 4 Effect of enzyme concentration on esterification Reaction conditions: Temperature: 37^{o}C, time: 72 hrs, agitation: 150rpm, acetic acid: butanol ratio1:0.5

Enzyme concentration.(%w/w)	% conversion
10	80
20	42.85
30	20

The result shows maximum % conversion of ester when 10 %(w/w) of enzyme was used and drastic decrease at 20% and 30%. The use of large amount of enzyme could significantly increase the fraction of acyl donar molecules to form acyl- enzymes complexes. Moreover, their active sites would not be exposed to the substrate and remain inside the bulk of enzyme particles without contributing significantly to the reaction. In the presence of high amount of lipase, not all active sides are exposed to the substrates and resulted molecules of the enzyme tend to aggregate together. However, too small amounts of enzyme may have been insufficient for complete substrate conversion within the specified reaction period.

3.6 Reusability:

The main advantage of enzyme immobilization is the reduced cost because lipase can be repeatedly used. Lipase was recovered after each reaction and reused for subsequent synthesis cycles. No significant decrease in the synthesis activity was observed for the first three cycles of the reaction. Nevertheless, the conversion yield decreased rapidly from the fourth use of the biocatalyst as shown in table 5. This could be a result of leakage of enzyme the treatments like washing, centrifugation which lead to formation of clumps due to less compression resistance of CLEAs which increase mass transfer limitations for substrate[17]or the denaturation of the enzyme by acetic acid after three reaction cycles.

Table 5 Effect of repeated use of lipases on conversion (%) for production of butyl acetate Reaction conditions: Temperature: 37^{o}C, time: 72 hrs, agitation: 150rpm, acetic acid: butanol ratio1:0.5, enzyme concentration: 10%

Cycle	% conversion
1	80
2	73.33
3	73.33
4	60

3.8 Analysis of synthesized Butyl acetate by FTIR and TLC

The analysis of butyl acetate produced was done by FTIR. The FTIR spectrum of butyl acetate is shown in fig.1. The esters produced by both enzymatic and chemical method were also confirmed on TLC

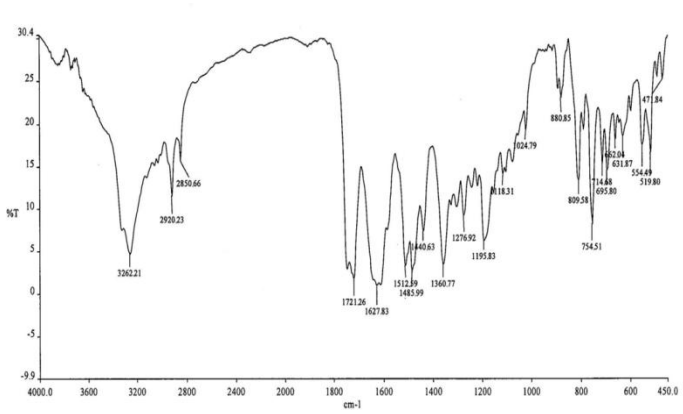

FIG 1 FTIR analysis of butyl acetate produced in lipase catalyzed esterification

The product butyl acetate is confirmed at wave number 1721 cm^{-1} . According to above fig. a strong peak was observed at 1721 cm^{-1} which indicated the presence of ester-carbonyl group (C=O). Also, peaks of CH_3 , CH_2 bending & C-O stretching vibrations were also observed at 3262 cm^{-1}, 2920 cm^{-1} and 1360 cm^{-}

4. CONCLUSION: Solid state fermentation with linseed oil cake and *Aspergillus oryzae* NCIM 1212 has been used for production of lipase. An enzyme activity of 6.248U/ml/min was obtained. This system was chosen for its greater applications in solid waste management and biomass energy conservation. The production of butyl acetate with a pineapple odour was achieved using immobilized lipase in the form of CLEAs. The maximum % conversion of butyl acetate (80%) was obtained at 37^0 C in 72 hours with the enzyme dose of 10% w/w; molar ratio of acetic acid to butanol, 1:0.5 in esterification.Also The stability of lipases in CLEA were found to be quite good. The enzymes were found to be stable and active for three repetitive cycles. This is a desirable property which can be useful for the economic production of food esters.

REFERENCES:

1. R. Aravindan , P. Anbumathi , T . Viruthagiri . *Lipase applications in food industry. Indian Journal of Biotechnology;* vol 6, 141-158. 2007.
2. H.F. Castro, P. C. Oliveira, C. M. F. Soares, G. M. Zanin, *Immobilization of porcein pancreatic lipase on celite for application in the synthesis of Butyl acetate in a non-aqueous system. JAOCS,* vol.76, 147-152. 1999.
3. F.J. Contensini, D. B. Lopese, G. A. Macedo, M. G. Nascimento, P. O. Carvalho, *Aspergillus sp. lipase: Potential biocatalyst for industrial use. Journal of Molecular Catalysis B: Enzymatic,vol* 67,163-171. 2010.
4. D. Das, V. Gunasekaran *Lipase fermentation: Progress and prospects. Indian journal of biotechnology*, vol 4, 437-445. 2004.
5. S. F. Dias, P. P. Cabral, M. M. R. Fonseca, *Modeling and production of ethyl butyrate catalyzed by Candida rugosa lipase immobilized in polyurethane foams. Biochemical engineering journal;* 148-158. 2007.
6. D. V. Gokhale, N. D. Mahadik, U. S. Puntambekar, K. B. Bastawde, J. M. Khire, *Production of acidic lipase by Aspergillusniger in solid state fermentation. Process biochemistry,* 715-721. 2002.
7. P. K. Ghosh, R. K. Saxena, Rani Gupta, R. P. Yadav, S. Davidson. *Microbial Lipases: Production and Applications, Science Progress,* 79(2); 119-157. 1996.
8. Helen Treichel, Déborade Oliveir, Marcio A. Mazutti, Marco Di Luccio, Vladimir Oliveira *A Review on Microbial Lipases Production. Food Bioprocess Technol*. 3: 182–196. 2010.
9. S. B. Imandi, , S. K. Karanam, , H. R. Garapati, *Optimization of media constituents for the production of lipase in solid state fermentation by Yarrowilipolyticafrom palm kernel cake (Elaeisguineensis). Advances in bioscience and biotechnology* .115-121. 2010.
10. Jean Louis Arpigny and Karl-Erich Jaeger, *Bacterial lipolytic enzymes classification and properties, Biochem. J.*; 343, 177–183. 1999.

11. K .Jaeger, M. Reetz, *Microbial lipases form versatile tools for biotechnology, Trends in Biotechnology;* 16: 396-403. 1998.
12. A. Kumar, S. S. Kanwar, *Lipase production in solid state fermentation (SSF): Recent devlopments and biological applications. Dynamics biochemistry; process biotechnology and molecular biology.* 2011.
13. S. Krishna, S. Divakar, Prapulla and, N. Karanth, *Enzymatic synthesis of isoamyl alcohol using immobilized lipases from Rhizomucor meichei, J. Biotechnol,* vol. 87, 193-201.2001.
14. S.M. Radzi, W. A. F. Mustafa, S. S. Othman, H. M. Noor, *Green synthesis of butyl acetate , a pineapple flavor via lipase catalyzed reaction. World academy of science, engineering and technology.* 2011.
15. A. Rajendran, A. Palanisamy, V. Thangavelu, *Lipase catalyzed ester synthesis for food processing industries. Brazilian archieves of biology and technology.* Vol 52, 207-219. 2009.
16. S. Sabat, V. K. Murthy, M. Pavithra, Pala Mayur, A. Chandavar, *Study of enhanced lipase production using agrowaste product by Bacillus stearothermophilusMTCC 37. International journal of pharmaceutical, chemical and biological sciences*. 266- 274. 2012.
17. R. Sheldon, *Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs), Appl Microbiol Biotechnol,* **92**,467–477. 2011.
18. S. Talekar, V. Shah, S. Patil, M. Nimbalkar, *Pourous cross linked enzyme aggregates (p-CLEAs) of Saccheromyces cerevisiae invertase, Catal Sci. Technol.* **2**,1575-1579, 2012.
19. L. Wilson, G. Lorente, R. Lafuente, A. Illanes, J. Guisan, J. Palomo, *CLEAs of lipases and poly-ionic polymers: A simple way of preparing stable biocatalysts with improved properties,* 2006.
20. R. Gupta, N. Gupta, P. Rathi, *Bacterial Lipases: An Overview Of Production, Purification And Biochemical Properties, Appl Microbiol Biotechnol*, 64, 763–78, 2004.