Research Journal of Recent Sciences _____ ISSN 2277-2502
Vol. **1(3),** 68-71, March **(2012)** *Res.J.Recent Sci.*

Short Communication

Synthesis of methyl cinnamate using immobilized lipase from *B. licheniformis* MTCC-10498

Sharma C.K. and Kanwar S.S.
Department of Biotechnology, Himachal Pradesh University, Shimla-171005, HP, INDIA

Available online at: www.isca.in
(Received 4th January 2012, revised 11th January 2012, accepted 12th Februry 2012)

Abstract

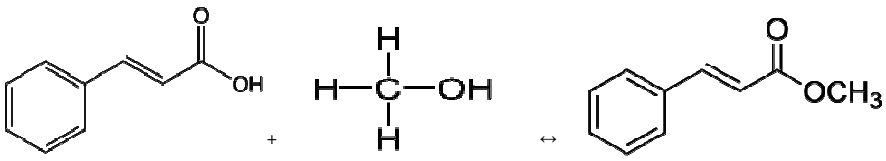
Methyl cinnamate was synthesized by the reaction of methyl alcohol with cinnamic acid using silica immobilized lipase from B. licheniformis MTCC-10498. The silica-bound lipase was used to perform esterification of methanol with cinnamic acid in equimolar ratio (100 mM each) in DMSO. Moreover, pure methyl cinnamate was used as internal standard. Methyl cinnamate was synthesized by manipulating various parameter i.e. relative proportions of reactants, reaction temperature, reaction time, concentration of molecular sieves and salt ions. Finally, 64.3 mM methyl cinnamate was synthesized at 65°C using 100 mM each of the reactants in 12 h in the presence of molecular sieves (100 mg/reaction volume) under shaking at 120 rpm. The immobilized lipase synthesized 50 % ester up to 5th cycle.

Keywords: *Bacillus licheniformis* MTCC-10498, immobilization, Silica, Formalin, Methyl cinnamate.

Introduction

Compounds of cinnamic acid are widely present in plants, including edible vegetable staple oil of cinnamon. Their biological properties, particularly the antioxidant activity, are well known and depend on the structural characteristics of these compounds[1]. Because of their relative polar properties, important efforts have been made in order to increase their hydrophobicity and therefore produce amphiphilic molecules of industrial value[2]. Interestingly, hydro-cinnamic esters have been used in the synthesis of HIV-1 protease inhibitors or as precursors for the synthesis of 1, 3, 4, 9-tetrahydropyrano [3, 4-b] indole-1-acetic acid, which is used as an analgesic, inflammation inhibitor and antipyretic compound[3]. Methyl cinnamate is a fragrance ingredient found in fragrances used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. Its use worldwide is in the region of 10–100 metric tones per annum. The maximum skin level that results from the use of methyl cinnamate in formulae that go into fine fragrances has been reported to be 0.31%, assuming use of the fragrance oil at levels up to 20% in the final product[4]. FEMA: Flavor and Extract Manufacturers' Association states: Generally recognized as safe as a flavor ingredient – GRAS 3. (2698)[5,6]

Biocatalytic methods are often preferred over chemical methods since their mild reaction conditions avoid unwanted side reactions, and decrease the possibility of producing pollutants[7]. Methyl cinnamate can be produced by esterification of cinnamic acid and ethanol catalyzed by a lipase (figure-1). Immobilized lipases generally offer economic incentives of enhanced thermal and chemical stability, ease of handling, recovery and reuse relative to non-immobilized forms. In the present study; we have synthesized Methyl cinnamate using silica-immobilized lipase in DMSO.



Cinnamic acid Methanol Methyl cinnamate

Figure-1
Synthesis of Methyl cinnamate using lipase

Material and methods

Chemicals: Methyl cinnamate (Hi Media, Mumbai, India); cinnamic acid , cinnammic acid, silica (S. D. Fine Chemicals, Mumbai, India); methyl alcohol, p-nitrophenyl palmitate (Alpha Aesar, Heysham, Lancs, England) and (Sarabhai Chemicals, Vadodra, India) were of analytic grade and were used as received.

Iimmobilization of *B. licheniformis* MTCC-10498 lipase on silica matrix: Swelling capacity of dry matrix (silica) was determined by incubating the preweighed (5 g) dry matrix in distilled water for 15 h at 8^{o}C. The swollen matrix was momentarily sedimented by centrifugation (10,000 g for 1 min). The aqueous phase was decanted completely. The silica was reweighed (g). The swelling capacity was found to be 1.16 times. The matrix was suspended in a solution containing purified lipase and the suspension was incubated for 1 h at 37^{o}C under shaking. The immobilized silica was activated by exposure to formalin (2%, v/v). The silica matrix was pre-treated with Tris-HCl 0.05 M, pH 8.5 to get rid of the excess formalin. The unbound protein was assayed for protein by Lowry's method and lipase activity by standard method. The silica-bound lipase was extensively washed with Tris-HCl (0.05 M, pH 8.5). The immobilized matrix was kept suspended in Tris-HCl (0.05M, pH 8.5) at 4^{o}C till further use. Its binding efficiency was 85.7 % and lipase activity was 1.21 U/mg.

Assay of lipase activity: The lipase was assayed by a colorimetric method[8] performed in triplicate. One unit (U) of lipase activity was defined as micromole(s) of p-nitrophenol released per minute by hydrolysis of pNPP by one ml soluble enzyme or 1 g silica bound enzyme (weight of matrix included) at 55^{o}C under assay conditions. Specific activity was expressed as µ mole(s) of the p-nitrophenol released per min by one mg of protein.

Application of immobilized lipase in synthesis of methyl cinnamate: The catalytic potential of immobilized lipase of *Bacillus licheniformis* MTCC -10894 was checked for the synthesis of methyl cinnamate by manipulating various physical and kinetic parameters.

Determination of amount of methyl cinnamate: A reference curve was plotted between molar concentration of ethyl cinnamate (20 to 100 mM) and the area under the peak (retention time 0.89 min). A sample size of 2 µl was used for GLC analysis. After the completion of esterification reaction at 65ºC specified time intervals (0-15 h, 120 rpm) the reaction mixture was withdrawn (2 µl) and subjected to analysis of methyl cinnamate by GLC (figure 2). The GLC (Michro-9100, Netel Chromatographs, and Thanne, India) was programmed for oven temperature 250ºC, injector 260ºC and FID temperature 270ºC. The detection of ethyl cinnamate was performed on 10% SE chromo WHP column (2 meter X 1.8 inch) using N_2 as a carrier gas at a flow rate 30 ml/min.

Synthesis of methyl cinnamate under optimized conditions: The immobilized matrix was washed twice, in 1 ml of DMSO (solvent at room temperature i.e. 20 ± 5 ºC. Thereafter the matrix was recovered by decantation of DMSO and used to catalyze the esterification of methyl cinnamate. The effect of concentration and relative molar ratio of methyl alcohol and cinnamic acid on the synthesis of methyl cinnamate was determined by keeping the concentration of one of the reactants (methyl alcohol or cinnamic acid) at 100 mM and varying the concentration of second reactant (25-100 mM) in a reaction volume of 1 ml in DMSO. The esterification was carried out using matrix bound lipase (20 mg) at 55ºC in 5 ml teflon coated screw cap tube for 12 h under continuous shaking (120 rpm). The methyl cinnamate formed in each of the combinations of the reactants was determined by GLC analysis.

Reusability of bound lipase on synthesis of methyl cinnamate: The formation of methyl cinnamate from cinnamic acid and methyl alcohol (100 mM: 100 mM) with bound lipase was assayed for 5th cycles of 12 h each. After each cycle of esterification, the bound lipase was washed twice for 5 min each in 1 ml DMSO at room temperature (25 ± 5 °C). Thereafter, DMSO was decanted and matrix was reused for fresh cycle of ester synthesis under similar conditions.

Results and Discussion

Synthesis of methyl cinnamate: The bound lipase was subsequently employed to synthesize ethyl cinnamate esters under optimized conditions in a water free organic solvent system. Ester synthesis in the water-free media/ organic solvents could be achieved very easily[7,9,10,11]. Since reactants /products are not soluble in n-alkanes, and were only soluble in DMSO. We have chosen the DMSO as a model solvent in the present study. The effect of various parameter *i.e.*, relative molar concentration (100:100 mM), biocatalyst load (20 mg), reaction temperature (55˚C), reaction time (12 h) and addition of molecular sieve (100 mg) had resulted into the synthesis of 63.4 mM of methyl cinnamate. A relative excess molar concentration of either acid or alcohol would denature or precipitate protein and such effect would inactivate the biocatalyst and thus would decrease the ester synthesis. Recently, we have reported that optimal synthesis of ethyl cinnamate at an equimolar proportion of reactants (100 mM each) in DMSO[7]. Esterification is generally a water-limited reaction because the equilibrium catalyzed by hydrolytic enzymes is in favor of hydrolysis[11]. Molecular sieves are an important class of synthetic adsorbents, which possess high porosity with pores of uniform size and essentially of molecular dimensions[12]. When molecular sieves were added in the reaction mixture, and the effect of

its concentration in the reaction system on rate of esterification by silica-bound lipase was studied, increase in the amount of ester was noticed. Thus, presence of molecular sieves in the reaction mixture was necessary to improve the esterification between ethyl alcohol and cinnamic acid.

Reusability of immobilized enzyme for ester synthesis: The bound lipase when repetitively used to perform esterification at 65^0C under optimized conditions resulted in 31.4 mM methyl cinnamate after 5th cycle of esterification.

Table-1
Reusability of bound matrix

Cycle No.	Methyl cinnamate synthesized
1.	64.4±2.3
2.	58.7±1.1
3.	51.3±1.8
4.	38.7±0.3
5.	31.4±1.3

Conclusion

The present study thus showed that silica immobilized-lipase efficiently performed esterification of methyl alcohol and cinnamic acid into methyl cinnamate at a temperature of 55^{o}C, addition of molecular sieves in conjunction with a silica matrix caused a increase in the rate of esterification; and an equi-molar ratio of ethyl alcohol as well as cinnamic acid was necessary for achieving an optimal rate of trans-esterification.

Acknowledgements

The financial grants in the form of a Research fellowship given by University Grants Commission, to one of the authors (Chander Kant Sharma), and by Department of Biotechnology, Government of India to Department of Biotechnology, Himachal Pradesh University, Shimla (India) are thankfully acknowledged.

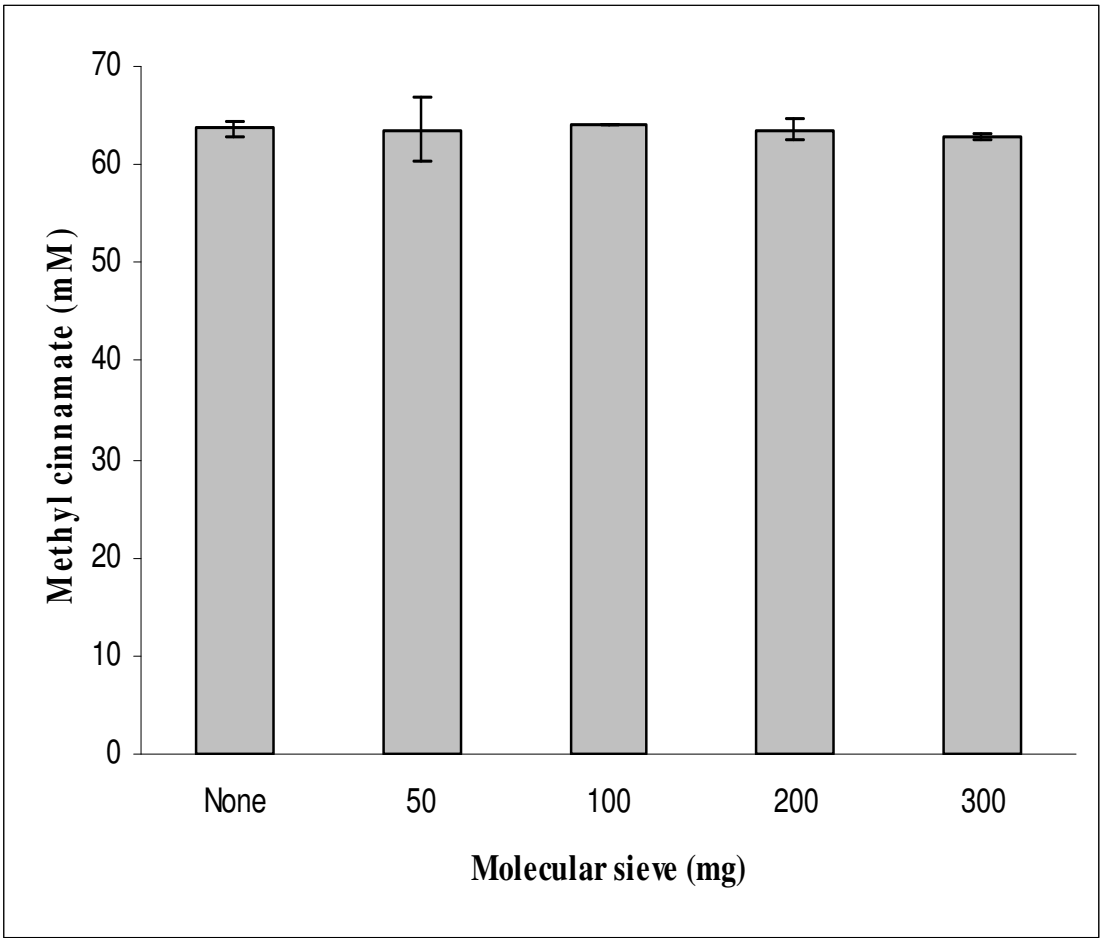


Figure-2
Synthesis of Methyl cinnamate using immobilized lipase under optimized controlled conditions (relative molar concentration (100:100 mM), biocatalyst load (20 mg), reaction temperature (55˚C), reaction time (12 h)

References

1. Nenandis et al., Radical scavenging potential of phenolic compounds encountered in *O. europaea* products as indicated by calculation of bond dissociation enthalpy and ionization potential values, *Journal of Agricultural and Food Chemistry,* **26**, 295–299 **(2004)**

2. Figueroa et al., Phenolic acids enzymatic lipophilization, *J. Agric. Food Chem.,* **53,** 2779- 2787 **(2005)**

3. Priya, Synthesis of Hydrocinnamic Esters by Pseudomonas cepacia Lipase, Enzyme and Microbial Technology, **32,** 485 **(2003)**

4. International Fragrance Association, *Use Level Survey*, **(2001)**

5. Flavor and Extract Manufacturers Association, *Recent progress in the consideration of flavoring ingredients under the food additives amendment III, GRAS Substances.* Food Technology, **19 (2, part 2),** 151–197 **(1965)**

6. Joint Expert Committee on Food Additives, *Safety evaluation of certain food additives, Who Food Additives Series: 46.Prepared by the Fifty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives.* World Health Organization, Geneva, **(2000)**

7. Sharma C.K. et al., Synthesis of medically important ethyl cinnamate ester by porcine pancreatic lipase immobilized on poly (AAc-co-HPMA-cl-EGDMA) hydrogel, *J Appl Polym Sci,* **121(5),** 2674-2679 **(2011)**

8. Winkler et al., Glucogen hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by Serratia marcescens, *J Bacteriol,* **138**, 663–670 **(1979)**

9. Kanwar S.S. et al., Short-chain ester synthesis by transesterification employing poly (MAc-co- DMA-cl-MBAm) hydrogel-bound lipase of Bacillus coagulans MTCC-6375, *J Appl Polym Sci,* **109,** 1063-1071 **(2008)**

10. Claon et al., Effect of reaction parameters on SP435 lipase catalyzed synthesis of citronellyl acetate in organic solvent, *Enzyme Microb Technol*, **16,** 835-838 **(1994)**

11. Halling, Thermodynamic predictions for biocatalysis in non-conventional media: Theory, tests and recommendations for experimental design and analysis, *Enzyme MicrobTechnol,* **16,** 178-206 **(1994)**

12. Pery R.H. et al., In Chemical Engineering Handbook, McGraw-Hill, New York, **16,** 2-11 **(1973)**