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APPLICATION OF LIPASE PRODUCED BY *PSEUDOMONAS AERUGINOSA* SJ2 IN THE SYNTHESIS OF METHYL SALICYLATE

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ABSTRACT

Purified lipase having specific activity of 2,826 U/mg produced by *Pseudomonas aeruginosa* SJ2 was used for the synthesis of methyl salicylate an ester. Initially a reference profile of standard methyl salicylate was prepared in DMSO for gas chromatography. Under unoptimized conditions lipase was able to convert 52.69 % of reactants into methyl salicylate. During optimization studies effect of amount of lipase, effect of relative amount of reactants, and incubation time was studied. Maximum methyl salicylate yield of 64.21% was observed when amount of enzyme was 20-30 µL. Maximum yield of 63.84% of methyl salicylate was achieved at a 1:1 molar ratio (50 mM:50 mM) of reactants and maximum yield of 67.05% was achieved at the end of 12h incubation period. When all the above parameters were optimized maximum conversion of 67.05% was achieved.

KEYWORDS: *Pseudomonas aeruginosa* SJ2, lipase, methyl salicylate, gas chromatography.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, E. C. 3. 1. 1. 3) are enzymes that catalyze the hydrolysis of triacylglycerols to fatty acids and glycerol. Lipases are found everywhere in nature and are produced by different species of plants, animals and microorganisms. Among the various lipases bacterial and fungal lipases are extensively used in biotechnological applications and organic chemistry. Bacterial lipases are mostly extracellular produced by several wild type and recombinant strains of bacteria viz., *Achromobacter, Alcaligenes, Arthrobacter, Archeglobus, Bacillus, Brochothrix, Burkholderia, Chromobacterium, Corynebacterium, Cryptococcus, Enterococcus, Lactobacillus, Microthrix, Moraxella, Mycobacterium, Pasteurella, Propionibacterium, Proteus, Psychrobacter, Staphylococcus, Serratia, Streptococcus, Sulfolobus, Vibrio* and *Pseudomonas*. Among these, the lipases from *Pseudomonas* bacteria are extensively employed for many different biotechnological applications (Jaeger et al., 1994; Pandey et al., 1999; Beisson et al., 2000). Lipases are used in food, cosmetics, biofuel, textile, leather, paper, pharmaceuticals, detergents etc. In addition to these industrial applications, lipases are also being investigated for their potential use in a variety of other areas, such as environmental remediation, bioremediation, and nanotechnology. Among the esters, methyl salicylate is a constituent of wintergreen and plants like *Amblyomma veriegatum* and *A. herbarium* used as a local anaesthetic agent and disinfectant also commercially used in toothpaste, mouthwash, perfumes and flavouring agents (Soares et al., 2005; Lusby et al., 1991). Methyl salicylate has various medicinal applications and is widely used to control muscular pain. Owing to applicability and importance the present work was focused on the synthesis of methyl salicylate using *P. aeruginosa* SJ2 purified lipase.

MATERIALS AND METHODS

Enzyme source

Pseudomonas aeruginosa SJ2 identified and characterized in our previous studies was used as a source of lipase. Crude enzyme obtained from *P. aeruginosa* SJ2 was purified by ammonium sulphate precipitation, DEAE Cellulose and Sephadex G-75 chromatography. Purified enzyme having specific activity of 2,826 U/mg was used in the present study.

Preparation of reference profile

A reference profile of methyl salicylate was prepared in DMSO and the corresponding area under the peak was determined. Synthesis of methyl salicylate was studied by taking 10µl of *P. aeruginosa* SJ2 lipase in the reaction mixture consisting of (50mM methanol: 50mM salicylic acid) in DMSO with the final volume of 2 mL. Reaction was carried at 40 °C for 8h under shaking at 120rpm. Control was prepared by adding heat inactivated enzyme (100 °C for 5 min). Methyl salicylate synthesized was determined by gas chromatography (GC) using a sample size of 1 µL. The GC was equipped with a capillary

column of 50 m length and internal diameter 0.32 cm. Nitrogen was used as a carrier gas at a flow rate of 1.5 ml per min. FID was set at 250 °C and injector temperature was set at 230 °C.

Optimization of reaction conditions

In optimization studies effect of the amount of lipase, relative concentration of the reactants and time of incubation were studied as below.

Effect of the amount of lipase

The synthesis of methyl salicylate was studied by taking different amounts of lipase enzyme (10, 20, 30, and 40 µL) in 2 mL of reaction mixture containing 50 mM each of methanol and salicylic acid in DMSO at 40 °C for 8h under shaking at 120rpm. Samples were withdrawn and analysed for methyl salicylate after 8h. Enzyme concentration that gave the best result was selected for further studies.

Effect of relative proportion of reactants

The effect of relative proportion of methanol and salicylic acid on synthesis of methyl salicylate was determined by keeping the concentration of one of the reactants, i.e., methanol at 50 mM and varying the concentration of second reactant salicylic acid (10, 30, and 50 mM) in a reaction volume of 2 mL in DMSO. The esterification was carried out in the presence of 20 µL of lipase at 40 °C under continuous shaking at 120rpm. Methyl salicylate formed in each of the combinations of the reactants was determined by GC analysis. Reactant concentration that gave the best result was selected for further studies.

Effect of incubation period

The reaction mixture (2 mL) contained 20 µL of lipase, 50 mM each of methanol and salicylic acid in DMSO. The reaction mixture was incubated at 40 °C under shaking at 120rpm up to 24h. The reaction mixture was sampled at an interval of 4h and subjected to analysis by GC for the formation of methyl salicylate.

RESULTS AND DISCUSSION

Reference profile of methyl salicylate prepared in DMSO (**Figure 01**) was used for the measurement of amount of methyl salicylate produced by lipase under different conditions. Under the unoptimized conditions lipase from *P. seruginosa* SJ2 was able to convert 52.69 % of the reactants into methyl salicylate.

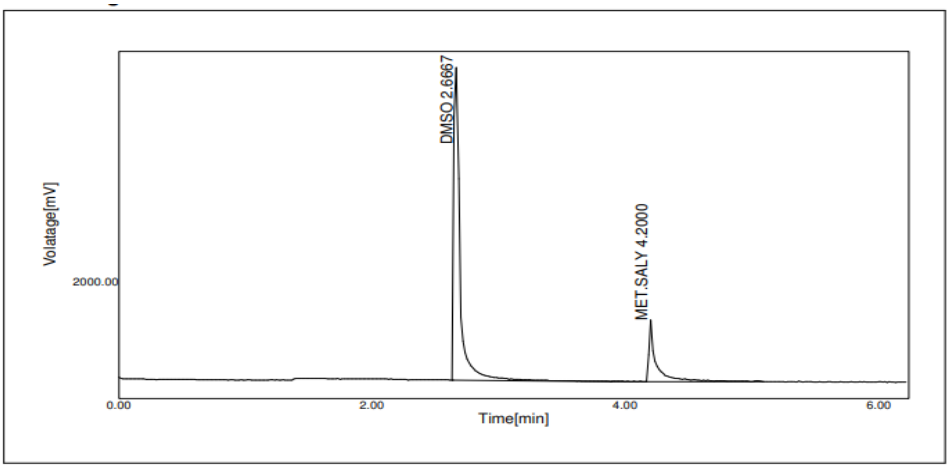

Figure: 01 GC report showing peaks of DMSO and methyl salicylate.

Optimization of reaction conditions

Effect of amount of lipase

Effect of amount of lipase was studied by varying the amount of lipase between 10-50 µL. Maximum methyl salicylate yield of 64.21% was observed at 20 and 30 µL volume of lipase while the molar conversion decreased when 40 µL lipase was used (**Figure 02**). Similar findings were reported by Kaur et al., (2019) where maximum yield of 86% of methyl butyrate was achieved at the concentration of 30 µg/mL of lipase isolated from *A. fumigatus* and concentration of lipase beyond 30 µg/mL decreased the amount of molar conversion. Upon increasing the enzyme amount further, the molar conversion was decreased which might be due to difficulty in maintaining uniform suspension of the biocatalyst at higher enzyme concentration. The excess enzyme did not contribute to the increase in the percentage conversion (Kaur et al., 2019). Bhardwaj et al. (2017) have reported 74.03% yield of methyl salicylate using immobilized lipase from *Geobacillus sp.* at 20 mg/mL of reaction volume concentration. In another study Kanwar et al. (2007) reported maximum synthesis of methyl acrylate with immobilized alkaline thermoalkalophilic extracellular lipase of *P. aeruginosa* MTCC-4713 at a concentration of 12.5 mg/mL.

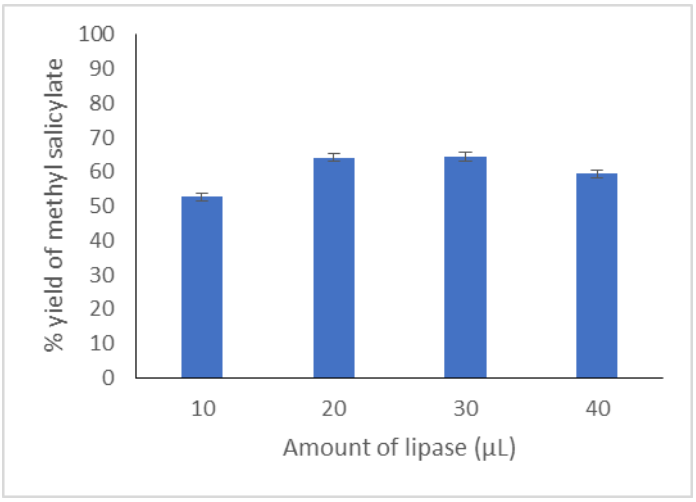

Figure: 02 Effect of amount of *P. aeruginosa* SJ2 lipase on synthesis of methyl salicylate.

Effect of relative concentration of the reactants

In the present study maximum yield of 63.84% of methyl salicylate was achieved at a 1:1 molar ratio (50 mM:50 mM) (Figure 03). The yield of methyl salicylate lowered at other molar ratios which may be attributed to either a steric hindrance or electronic effect of substrate on the purified lipase or specificity of purified lipase toward the substrate (Kaur et al., 2019). Similar results were reported by Kaur et al. (2019) where a maximum yield of 81.4% of methyl butyrate was achieved with a 2:2 molar ratio of methanol and vinyl butyrate in the presence of lipase isolated from A. fumigatus. Bhardwaj et al., (2017) have reported 70.25% yield of methyl salicylate at the molar ratio of 2:4 of salicylic acid and methanol respectively in the presence of immobilized lipase isolated from Geobacillus sp. Martínez-Ruiz et al., (2018) have reported a maximum yield of 93.4% of methyl butyrate at 1:1 molar ratio of methanol: vinyl butyrate in the presence of immobilized lipase isolated from C. antartica. de Souza et al. (2018) have reported a maximum yield of 90% of methyl oleate at the molar ratio of 1:2 of oleic acid: ethanol in the presence of lipase isolated from R. microsporus.

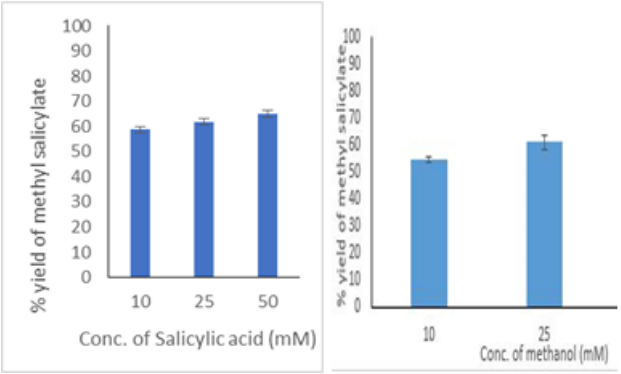

Figure: 03 Effect of relative concentration of reactants on synthesis of methyl salicylate.

Optimization of reaction time

The maximum yield of 67.05% was achieved at the end of 12h incubation after which there was a decline in conversion rate (**Figure 04**). Similar results were reported by Bhardwaj et al., (2017) where 12h incubation was found to be optimum for the maximum yield of 73.07% of methyl salicylate in the presence of immobilized lipase isolated from *Geobacillus sp.* Kaur et al. (2019) reported optimum incubation period of 16h for the maximum yield of 84.2% of methyl butyrate in the presence of lipase isolated from *A. fumigatus*. Kumar et al. (2006) reported **t**he optimum incubation time of 12h for the synthesis of ethyl propionate using silica bound lipase from *Bacillus coagulans* BTS-3. Raghuvanshi and Gupta (2009) reported optimum time of 3h for immobilized lipase from *B. coagulans* BTS-3 for synthesis of *p*-Nitrophenyl acetate. Garlapati and Banerjee (2013) reported optimum incubation period of 14h for the synthesis of methyl butyrate using immobilized lipase from *Rhizopus oryzae* NRRL 3562.

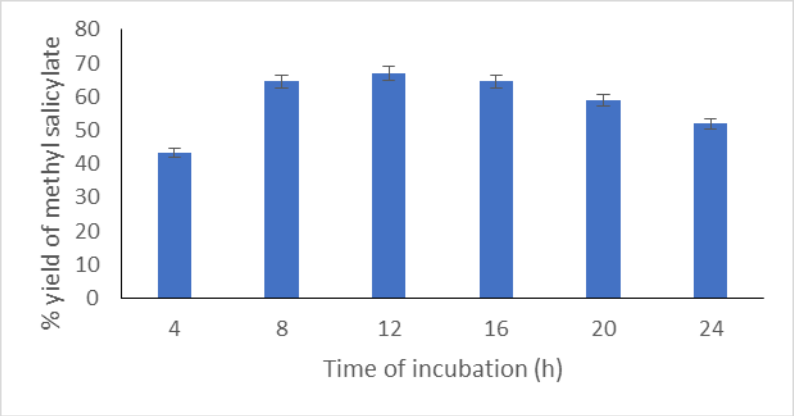

Figure 04: Effect of reaction time on synthesis of methyl salicylate.

CONCLUSION

Lipase having specific activity of 2,826 U/mg was used for the synthesis of methyl salicylate. Under optimized conditions, lipase from *P. aeruginosa* SJ2 was able to achieve 67.05% of substrate conversion. In future synthesis of other esters will be conducted and scale-up studies will be also be performed to detect the efficiency of *P. aeruginosa* SJ2 lipase.

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