Synthesis of Bioflavor (Butyl Acetate) Using Bacterial Lipases

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Abstract- *Lipases have recently emerged as key enzymes in the swiftly-growing biotechnology sector. Lipases carry out two important functions- firstly breakdown of lipids involving hydrolysis of triglycerides into glycerol and free fatty acids which is used in Dairy, Oil industries and can be used in Bioremediation. Secondly Lipase-catalyzed esterification reactions are among the most significant chemical and biochemical processes of industrial relevance which are used in ester production.Six bacterial strains were selected from soil as potential lipase producers among which four were selected as good lipase producer by lipase assay. Further application of lipases extracted was done in green synthesis of Butyl acetate and estimated using FTIR that showed peak at 1742cm^{-1}confirming ester formation. Titrimetric estimation of the reaction mixture indicating highest amount of Butyl acetate was obtained with lipase extracted from C-1 culture (20.51%). The C-1 culture was sequenced using MALDI and was found to be Bacillus megaterium.*

Keywords- Butyl acetate, FTIR, MALDI, Bacillus megaterium

I. INTRODUCTION

Enzymes play a dynamic role in our day to day life. Lipases (Triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze hydrolysis of Mono-, Di- and Tri-acylglycerides into glycerol and free fatty acids in presence of water-lipid interface. **(1)**Microorganisms producing lipases are found in varied habitats such as industrial wastes, vegetable oil treating factories, soil contaminated with oil, oil seeds and decaying food, compost heaps ,coal tips and hot springs. **(3)**Even if lipase enzyme are isolated and purified from fungi, bacteria, yeast, animal and plant sources regardless of it, bacterial lipases are considerably commercially important and physiologically significant.**(1)**Most commercially useful lipases are of microbial origin. Due to commercial importance of extracellular lipases, many microorganisms have been studied for their lipase production ability. **(2)**

Most of the well studied microbial lipases are inducible extracellular enzymes. **(4)** Lipase enzymes are widely used in Bio-industries viz Cosmetics, Pharmaceutical, Dairy and Food. Lipases also show trans-esterification reaction that leads to the formation of esters.Green chemistry is the design of chemical products and processes to decrease or eliminate the use and generation of unsafe substances.Biocatalyst is one of the vital pillars thatfulfills the green chemistry concept.Lipase-Catalyzed esterification is most noteworthy chemical and biochemical process of industrial applicability. These esters can be used in the Bio-industries as they have fruity odours. Flavour compounds that are extracted from plants are too expensive and hence are replaced by flavor esters synthesized using catalysts. Flavor or fragrance materials which consist of various aliphatic and aromatic compounds share a major market of food additives throughout the world. Flavor esters are low molecular weight compounds produced by the esterification of fatty acids, preferably by microbial lipases. These compounds carry the tag natural, despite being synthesized and hence are a subject of intensive research. **(5)** Some of the esters synthesized by esterification reactions catalyzed by bacterial lipases are ethyl acetate, ethyl butyrate, ethyl methyl butyrate, ethylvalerate and ethyl caprylate. **(6-7)**

II. MATERIALS AND METHODS

In previous studies 6 lipase producing isolates were isolated from soil sources using Tributyrin agar in which 4 isolates were found to produce good amount of lipase activity on performing lipase assay. Further these 4 isolates were used for Bioflavor production. The 4 isolates were named C-1, C-2 , C-3 and C-4 respectively.

- **Synthesis of Butyl acetate**

Reaction mixture for Butyl acetate formation was run which consisted 50mmol of n-butanol and 50mmol of butyl acetate and 1ml of crude lipase enzyme was added from all the 4 selected isolates. All substrates were placed in conical flask which was cotton plugged. The flasks were kept at 37^{o}C for 24 hours in incubator shaker.

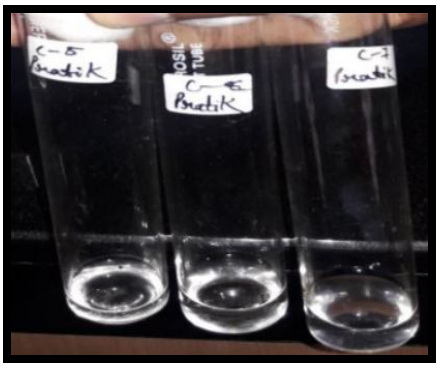

Fig-1 Reaction Mixture

- **Identification of Butyl acetate**

Preliminary detection of butyl acetate was done using Thin layer chromatography (TLC). 100% Petroleum ether was used as a solvent. The iodine fumes were used as a developing agent which turned butyl acetate spots to brown color. The synthesized butyl acetate was analyzed using spectroscopy methods of Fourier transform infrared spectroscopy (FTIR) to confirm the product obtained.

- **Analysis of Butyl acetate**

The quantification of the butyl acetate was done by simple acid-base titration using 0.1M NaoHand 0.1% phenolphthalein as an indicator. The yield of butyl acetate produced by the enzyme was expressed as a percent (%) of converted acetic acid as compared to the total fatty acid in the reaction mixture using the following equation:

$$\text{Conversion (\%)} = \frac{\text{Vol}_{\text{NaoH}}(\text{w/o enzyme}) - \text{Vol}_{\text{NaoH}}(\text{with enzyme})}{\text{Vol}_{\text{NaoH}}(\text{w/o enzyme})} \times 100\%$$

Formula For calculation

In this study, the butyl acetate produced was calculated in terms of relative percentage conversion (%).

- **Sequencing of the Isolate producing highestamount of Butyl acetate**

Sequencing of the isolate producing highest amount of Butyl acetate was achieved using matrix assisted laser desorption/ ionization- Time of Flight (MALDI-TOF), it was done at National center for cell sciences Pune, Maharashtra , India.

III. RESULT AND DISCUSSION

1. Synthesis and identification of Butyl acetate

On incubating the reaction mixture of n-butanol and acetic acid, the presence of butyl acetate was determined by TLC and FTIR analysis.

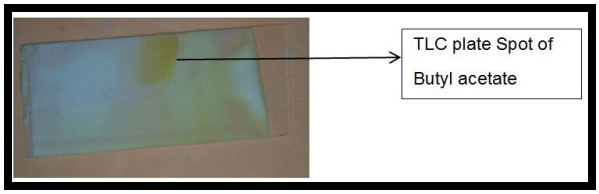

Fig-2 Preliminary Analysis – TLC

Brown color spot detected the presence of ester in the reaction mixture.

FTIR – Laboratory report of Dr P.S. Ramanathan Advanced instrumentation Centre

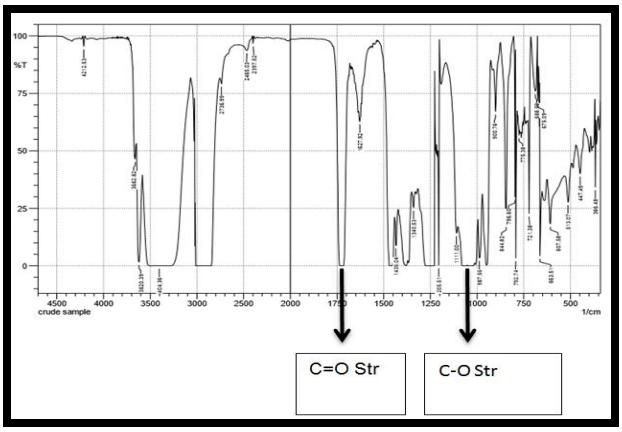

Fig-3 FTIR analysis.

C=O stretch and C-O stretch confirmed the presence of Butyl acetate in the reaction mixture. According to Fig- 3 a strong peak was observed at 1742 cm^{-1} which showed the presence of ester-carbonyl group. Similar results were found in**S. Mat Radz*iet al,*(2011)**wherein the butyl acetate was detected as brown spots on the silica gel plate when visualized by an iodine reagent. The synthesized butyl acetate was analyzed wherein FTIR, a strong peak was observed at 1742 cm^{-1} which indicated the presence of ester-carbonyl group**(8)**. Further in another study also, the presence of the oleyloleate, was detected as brown spots when visualized by an iodine reagent. Further identification was carried out by FTIR which showed a characteristic absorption of ester bond at 1742 cm^{-1}. Final identification of reaction mixture was performed by Gas Chromatography (GC) by comparing the ester with a known authentic standard.(**Mat Radz*iet al,* 2005) (9).**

2. Analysis of Butyl acetate

Analysis of the reaction product was done using simple acid-base titration using 0.1M NaoH and 0.1% phenolphthalein as an indicator, the endpoint was from colorless to pink.

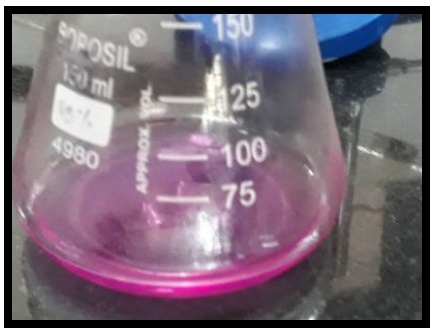

Fig-4 Titration of the Reaction mixture

Graphical representation of the Butyl acetate produced by all the 4 isolates.

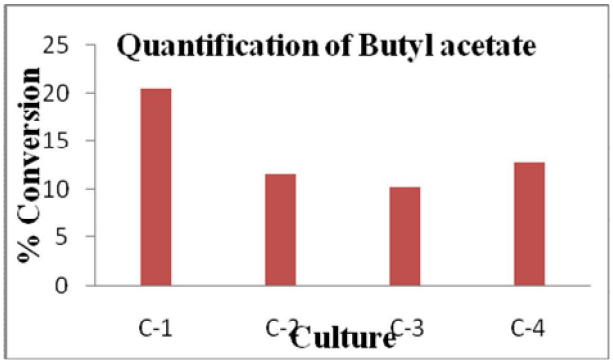

Table-1

C-1 showed highest amount of Butyl acetate production which was 20.51% on performing titrimetric method.In a similar study Novozym 435, *Candida antarctica* lipase B immobilised on a macroporous acrylic resin, Kojicmonooleate ester (KMO) was synthesized by mixing kojic acid, oleic acid and lipase powder (Novozym 435) in a 150 mL beaker without using any solvent. Analysis of the ester produced was done using Gas Chromatography which showed that the product yield was found to be 42.09%.**(KhairulazharJumbri*et al,* 2015) (10).** In another study, analysis of the reaction product was done by titrimetric method. Analysis of yield showed that under optimum conditions, >78 % of butyl acetate was produced using commercial immobilized lipase from *Rhizomucormiehei*(Lipozyme RMIM). **(S. Mat Radzi*et al,* 2011) (8).**

- **Sequencing of isolate producing Highest amount of Butyl acetate**

Amongst the 4 selected isolates for butyl acetate production C-1 showed highest amount of product formation which was found to be 20.51%. Therefore C-1 isolate was selected for sequencing that was done using MALDI-TOF. On sequencing the isolate was found to be *Bacillus megaterium.*

Laboratory report- MALDI TOF analysis from National center for Cell Sciences

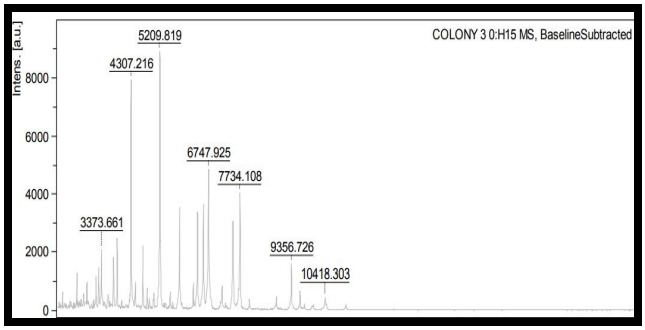

Fig-5 MALDI TOF analysis

On performing the MALDI TOF analysis of the culture showing maximum Butyl acetate production, it was found to be *Bacillus megaterium* as it showed high score value (2.173) to *Bacillus megaterium*DSM 2894. Similar results were found in another study where they have used other sequencing methods, wherein DOD9 isolated from Doddawad oil refineries was sub-cultured on nutrient agar slants and used for further experimentation. This lipase producing micro-organism was characterized as gram positive, spore forming, halophillic, mesophilic, motile, aerobic and showed positive results to catalase test, casein hydrolysis and starch hydrolysis. According to the observations it was classified as *Bacillusspp*.(**Pooja K Mahale*et al,* 2015) (11).** In another studythe bacterial isolate which showed maximum lipase production was further characterized and identified by morphological, and biochemical characteristics and by 16srRNA sequencing as *Pseudomonas gessardii*. **(M. Veerapagu*et al,* 2013) (12).**

*Bacillus megaterium*is being studied to produce lipase enzyme that has also being optimized. **(FadahunsiIllesanmi Festus *et al,* 2017) (13).** *Bacilliusmegaterium* AKG-1 was also found to produce thermostable lipase during submerged fermentation. **(AnuragSekhon*et al,* 2006).** But till now the lipase enzyme from *Bacillus megaterium*has not being studied in ester production.

IV. CONCLUSION

Esterification was carried out to produce Butyl acetate using the crude lipase enzyme obtained from *Bacillus megaterium*.Thus using this culture Green biological synthesis of bioflavourscan be employed as the replacement of chemical methods that can lead to various hazards. This method is eco-friendly, rapid and cost-effective.

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