MICROBIAL PRODUCTION OF MENTHOL FOR ENANTIOSELECTIVITY TOWARDS *L*-MENTHOL

BHATHENA Z. P, REVANDKAR SHITAL

Abstract: Enzymatic preparation of *l*-menthol has been attracting much attention in the flavor and fragrance industry. Two gram negative strains, labelled as BG-1 and VM-3, exhibiting high hydrolytic activity and excellent enantioselectivity towards *l*-menthyl ester, was successfully isolated from soil samples through enrichment culture techniques. The esterase enzyme extracted from isolate VM-3 caused enantioselective hydrolysis of 100 mM *d*/*l* menthylacetate at 30°C and pH 7.0, resulting in 99% selectivity for the *l*-menthol, which were much better than those obtained using commercial esterase enzymes. Moreover, it exhibited strong tolerance towards high substrate concentration (up to 500 mM), without any feed- back inhibition due to accumulation of the *l*-menthol. These results imply that our isolate can be a promising biocatalyst for the large-scale enzymatic preparation of *l*-menthol due to the use of mild, efficient, inexpensive and easy-to-use "green chemistry methodology".

Keywords: Enzyme catalysis; high substrate concentration tolerance; hydrolysis; l-menthol; *dl*-menthyl acetate.

Introduction: *l*-menthol has been attracting much attention in the flavour and fragrance industry as it is one of the world's largest flavour component and is widely used in many confectionary goods, pharmaceuticals, oral health care products, cosmetics, teas and tobacco products due to its pleasant flavour , aroma and its cooling-anaesthetic effect [1]. The characteristic cooling sensation of *l* menthol is produced by interaction with cold receptors rather than the taste buds, thus its manifestation is not limited to the oral cavity. Menthol produces the sensation of coolness in the oral and olfactory regions only at low concentrations, as higher concentrations it induces a burning sensation coincident with some modest degree of desensitization [2].

Menthol has been traditionally extracted by steam distillation from plants, *Mentha piperita* and *Mentha arvensis* belonging to Labiatae family as this family is well known for accumulating aromatic volatile oils within its foliage. But such extraction leads to about 50% recovery of the oil that contains *l, d , d*/*l* isomers and neomenthol. The *d- and l*-isomers of menthol each have identical physical properties apart from their specific optical effect on rotation of light. But, *d*-menthol adds undesirable taste and thus does not find acceptability for human use. Additionally, *d*-neomenthol is a colorless liquid while isomenthol and menthol are white crystals. [3]. Thus the mentha plant oils needs to be to obtain the widely used *l*-menthol, by removing the undesirable *d*-menthol isomer.

Additionally, it only *l*-menthol that is approved by FDA with GRAS status amongst the category of essential oils, oleoresins, and natural extractive and has approval for use as a synthetic flavouring substance and adjuvant in foods with

no limitation on usage except good manufacturing practices [4]

Thus, a bio catalysis method that can help to attain enantioselectivity will enable a harvest of *l*-menthol that has better purity One way of preparing *l*-menthol is enantioselective ester hydrolysis or transesterification of racemic menthol in organic solvents mediated by lipases or esterases. Microbial enzymes like lipases are capable of acting on alcohol racemates through hydrolysis or condensation of the corresponding esters. Such lipase catalyzed reactions have become particularly popular because lipases are readily available from commercial sources, are relatively inexpensive, have no co-factor requirement, and thus are widely acceptable in industry. In kinetic enantiomeric resolution reactions, lipases can effectively transform the ester substrate into *l*-menthol without converting it into the corresponding *d*-enantiomer, however its actual industrial application has been restricted due to its low esterification rate and low reactivity of acyl donor. [5]-[6].

Thus though, theoretically l-menthol can be obtained through enantioselective hydrolysis of racemic menthyl esters in aqueous medium mediated by a specific hydrolase, very few examples of the preparation of *l*-menthol in this manner have been reported so far. This combined with their low activity and low substrate concentration tolerance have restricted the application of enzymes and microorganisms for industrial application

Thus, a screening programme for organisms that produces lipase/ racemases that can enable enantioselectivity can help to readdress the requirement of *l*-menthol. Screening of microorganisms for their hydrolytic enzymes like lipase/esterase enzyme would be based on their high selectivity, high specific activity and strong tolerance against substrate/product concentration. Thus the aim of this study was to enrich and isolate organisms capable of producing enzymes that enable the production of menthol and enantioselective separate the bioactive *l*-menthol.

Materials & Methods:

Enrichment and screening of microorganisms: Different soil samples were collected from various regions of Mumbai such as Bhavan's garden, Borivali National park, Versova mangrove, compost soil, Mahim garden, and Salaskar garden Jogeshwari. Collected soil samples were serially enriched using increasing concentration of *dl*-menthyl acetate (10 mM & 100 mM) in a minimal medium containing (per liter): K_2HPO_4, 2.0 g; KH_2PO_4, 7.0 g; $MgSO_4$, 0.1 g; and $Na_3C_6H_5O_7$, 0.5g; pH-7. Enrichment was carried out in 250 ml flask with 50ml of medium at RT for 7 days with shaking at 180 rpm.

Primary screening of microorganisms [7]**:** The primary screening of the isolates was undertaken to assess the hydrolytic ability of isolates using *dl*-menthyl acetate hydrolysis agar medium. Semi-quantitative method was used in which 24 hrs old actively growing strains were adjusted to 0.1 O.D. at 530 nm using saline and 20 µl of the culture suspension was spotted on *dl*-menthyl acetate agar medium and then incubated at RT for 48 hrs. The strains having opalescent zones around them, indicating hydrolysis of *dl*-menthyl acetate to menthol were selected after incubation at RT for 48 hrs and activity ratio was determined. Activity ratio was determined as the ratio of diameter of the zone of opulence observed due to *dl*-menthyl acetate hydrolysis over the diameter of the colony grown in medium containing 100 mM *dl*-menthyl acetate. The isolates having highest activity ratio were selected for secondary screening.

Secondary screening [8]**:** The isolates obtained after the primary screening process were incubated in minimal based medium (per liter):

K_2HPO_4, 2.0 g; KH_2PO_4, 7.0 g; $MgSO_4$, 0.1 g; and $Na_3C_6H_5O_7$, 0.5g; pH-7 containing 100 mM *dl*-menthyl acetate at an inoculum density 5% and flasks were incubated under shaker conditions (REMI Pvt Ltd) at 180 rpm in RT for 48 hrs. The metabolic filtrate obtained by centrifuging at 3000 rpm for 10 mins was treated with equal volume of ethyl acetate to extract menthol in the organic phase and analyzed by thin layer chromatography.

Analytical methods:

Thin layer chromatography [8]**:**

TLC analysis was carried out on RSG F254 silica gel sheets using mobile phase of petroleum ether and ethyl acetate in a ratio of 10:1 (v/v). An aliquot of 5 µl of extracted menthol along with standard menthol (0.01%) (Sigma) was spotted on the plate and plate allowed to run for 8 cm. Spots were visualized by treatment with 5% (w/v) vanillin/ concentrated sulphuric acid solution.

Preparation of esterase enzyme: The isolates (strain BG1 and VM3) used at inoculum density of 5% were grown aerobically in 100 ml of minimal medium containing *dl*-menthyl acetate (100 mM). The medium was incubated at 30°C for 24-48 hrs with shaking at 180 rpm. The cells were then centrifuged at 10000 x g for 10 min, washed twice with physiological saline and the harvested biomass was resuspended in 50 mM phosphate buffer pH 7. The biomass was disrupted using a sonicator (LABSONIC M) at 8 pulses/min using 80% amplitude. The cell debris was removed by centrifugation at 10000 *g* for 3 mins and the resultant supernatant used as the esterase extract.

Determination of esterase activity [9]**:** Esterase activity was measured using p-nitophenyl butyrate (pNPB) as a substrate. 50 µL of the above prepared enzyme extract was added to 2.92 mL of 100 mM sodium phosphate buffer (pH 7.0). After preincubation at 30°C for 3min, the reaction was initiated by a quick mixing of the reaction mixture with 30µL of p-nitrophenyl butyrate (100mM) solution prepared in dimethyl sulfoxide (DMSO).The reaction was stopped after incubation at37°C for 15 mins by addition of 4 ml of 0.25N NaOH. The absorbance was determined at 405 nm due to release of p-nitrophenol**.** One unit of activity was defined as the amount of enzyme releasing 0.001 mM of p-nitrophenol per minute under such conditions. Amount of p-nitrophenol released was determined using standard p-nitrophenol assay prepared within range of 5-50 mM p-nitrophenol **.**

Enantioselective hydrolysis of *dl*-menthyl acetate for harvest of *l*-menthol [10] **:** A reaction mixture consisting of 1.8 mL of 0.202 mM of dl-menthyl acetate prepared in phosphate buffer pH 7 and 0.2 ml of ethanol was treated with 20 µl of esterase enzyme obtained from shortlisted isolates, in an attempt to detect the enantioselective *l*-menthol formation. Additionally, whole cell biomass suspensions of shortlisted isolates instead of esterase enzyme were also mixed with *dl* menthyl acetate in order to assess the ability of intracellular enzyme to form enantioselective *l*-menthol. Both the reaction mixtures were then incubated at shake conditions at 30°C for 48 hrs. The product thus formed was extracted using equal volume of ethyl acetate. The reaction mixture was centrifuged at 3000 *g* for 10min and the *l*-menthol extracted in organic upper layer detected using 5% vanillin sulphuric acig reagent. The enantiomeric excess of the product due to the conversion of the substrates were determined by chiral GC analysis. Commercial enzymes (Lipase D, Lipase PS,) were also used to determine their hydrolytic activity towards *dl*-menthyl acetate in similar conditions as mentioned above.

The ability of the isolates to undertake enantioselective hydrolysis of *dl*-menthyl acetate

at a higher concentration of 500 mM was also set up in the aim to assess the effect of variation of substrate concentration using both enzyme and biomass extract.

Chiral GC Analysis [2]: The optical purity and conversion were determined by GC-14 gas chromatography equipped with FID detector and Cydex-B column (0.25 micron film 50m X 0.22mm I.D) using nitrogen as carrier gas using n-octanol as an internal standard. The injector and detector temperatures were set at 240°C and 300°C, respectively. The initial column temperature of 100°C was held for 5 min, then raised to 130 °C at a rate of 2°C/min and finally held at 130°C for 10 min. Optically pure menthol (Sigma) with retention times: *d*-menthol, 15.6 min; *l*-menthol, 15.9 min were also run. The enantiomeric excess of product obtained with the hydrolytic mixture extracted using ethyl acetate was calculated according to Chen et al (1982) using following formula

$$\text{Enantiomeric excess of l-menthol} = \frac{[l]-[d]}{[l]+[d]}$$

where *[l*] and [*d*] represent concentration of *l* and *d* menthol respectively in product extracted.

Results & Discussion:

Enrichment and screening of microorganisms: The search for novel biocatalyst involves exploring various natural ecosystems with the aim to obtain microbes that have ability to metabolize and/or bio-transfrom a wide variety of natural and synthetic organic compounds through their enzymes. Hence, various soil samples were assessed for their microbial load that had the capability of hydrolysis *dl* menthyl acetate through serial enrichment technique using a synthetic medium containing increasing concentration (10mM & 100mM) of *dl*-menthyl acetate as the sole carbon source. The strains capable of hydrolyzing *dl*-menthyl acetate were detected on *dl*-menthyl acetate (100 mM) hydrolysis agar medium [Table 1] as colonies showing opalescent zone of hydrolysis of *dl*-menthyl acetate and their activity ratio was determined.

Primary screening of microorganisms: 18 isolates showing different morphological characteristics were found to be able to grow in presence of 100mM *dl*-menthyl acetate; with the most of the isolates obtained from the mangrove soil sample, the result of which shown in Table 1. Though these isolates were found to capable of growing in presence of *dl* menthyl acetate, only 50% (9 of 18 isolates) were found to be able to hydrolyze menthyl acetate such that opalescent zone of hydrolysis could be detected. Additionally, through Versova mangrove soil yielded the highest number of isolates capable of growing in presence of *dl*-menthyl acetate, these isolates showed poor hydrolytic capabilities in comparison to isolates obtained from National park soil from which 3 of 4 strains isolated were found to have hydrolytic capabilities of *dl*-menthyl acetate after 48 hrs. of incubation. Thus based on the activity ratio, isolates were shortlisted for further studies.

Secondary screening: Analysis of the isolates for further shortlisting potent strain was carried out using TLC analysis of the enantioselective hydrolysis reaction mixture. Out of 9 isolates, 2 isolates (NP-5 and VM-6) did not have ability to convert *dl*-menthyl acetate into menthol and thus isolates BG-1, BG-7, VM-3, SG-1, CS-1, and NP-3, were selected for further studies. Though NP-1 fraction on TLC showed reaction with vanillin, it resolved at an different Rf value different than the standard *l*-menthol and thus was not selected for further studies. 2 isolates NP-5 and VM-6 although showing good activity ratio were found unable to react with vanillin indicating that they were unable to convert *dl*-menthyl acetate to menthol . Such positive reaction on *dl*-menthyl acetate hydrolysis agar may be due to its hydrolysis products other than *l*-menthol. 3 isolates BG-5, SG-2 and CS-6 that did not show any activity ratio on *dl*-menthyl

acetate agar plates were used as negative controls (Table 2).

Zheng *et al* (2009) carried out similar screening experiments to isolate various microbial strains from soil samples through enrichment culture using *dl*-menthyl acetate as the sole carbon source and obtained 8 strains capable of producing optical pure *l*-menthol from the selected 265 active strains isolated in the presence of a lower concentration (10 mM) of the substrate

Production of esterase enzyme and determination of its activity: Screening of the shortlisted isolates quantitatively for their esterase enzyme based on ability to release p-nitrophenol from substrate p-nitrophenyl butyrate was performed. Esterases hydrolyze ester substrates with short-chain fatty acids (≤C10) [1]. Hence p-nitrophenyl butyrate, an

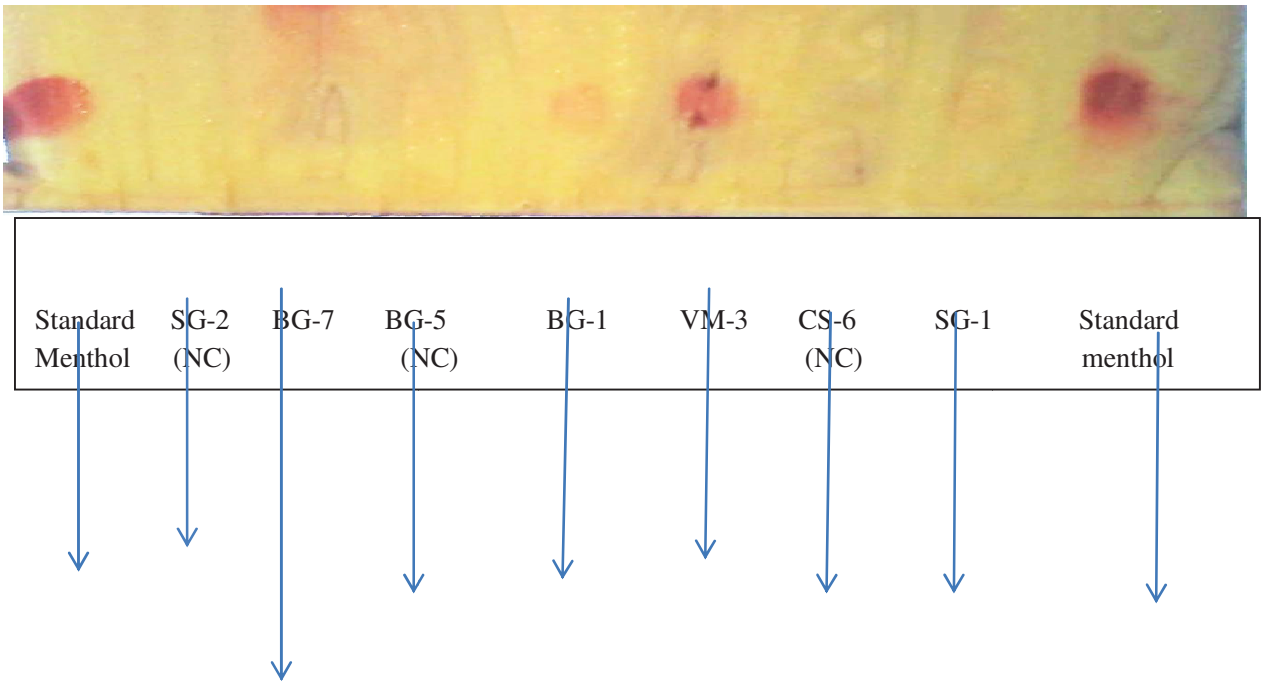
Table 1 Primary screening of isolates capable of *dl*-menthyl acetate hydrolysis

Sample	No. of isolates per soil sample	Strain number	Colony characters of the isolate	Activity ratio
Bhavan's garden soil	4	BG-1	Small, circular (1-2mm diameter), Off-white, opaque colony	**1.25**
		BG-3	Large, irregular, white, translucent colony	**-**
		BG-7	Small, irregular, brownish, translucent colony	1.12
		BG-5	Pinpoint, opaque, white coloured colony	-
Versova mangrove soil	5	VM-1	Large, irregular, white, translucent colony	**-**
		VM-3	Large, irregular, off-white, opaque colony	**1.3**
		VM-6	Large, circular (2-3mm diameter), Off-white, opaque colony	1.09
		VM-8	Small, circular (1-2mm diameter), Off-white, translucent colony	-
		VM-9	Pinpoint, opaque, white coloured colony	-
Salaskar garden soil	3	SG-1	Large, irregular, off-white, opaque	**1.27**

		SG-2	Pinpoint, opaque, white coloured colony	-
		SG-5	Small, circular (1-2mm diameter), Off-white, opaque	-
Compost soil	2	CS-1	Small, circular (1-2mm diameter), Off-white, opaque	1.14
		CS-6	Pinpoint, translucent, off-white coloured	-
National park soil	2	NP-1	Large, circular (2-3mm diameter), Off-white, opaque	1.18
		NP-3	Pinpoint, opaque, white coloured colony	1.08
		NP-4	Large, irregular, off-white, opaque	**-**
		NP-5	Small, circular (1-2mm diameter), Off-white, opaque colony	**1.23**
Total	18			

Table 2 Confirmation of menthol production using TLC analysis

isolates	**Retention factor**	**Inference**
BG-1	0.48	Menthol producing activity confirmed
BG-7	0.57	Menthol producing activity confirmed
BG-5	0.5	No menthol production
VM-3	0.47	Menthol producing activity confirmed
VM-6	-	No menthol production
SG-1	0.47	Menthol producing activity confirmed
SG-2	-	No menthol production
CS-1	0.48	Menthol producing activity confirmed
CS-6	-	No menthol production
NP-1	0.45	Menthol producing activity confirmed
NP-3	0.48	Menthol producing activity confirmed
NP-5	-	No menthol production
Std. menthol	0.48	



Standard menthol at 0.01% was used as a positive control while metabolic filtrate of isolates ***BG-5, SG-2, and CS-6*** *used as a negative control having no any Cx ratio on dl-menthyl acetate agar plate and it showed no reaction with vanillin on TLC plates thereby confirmed their inability to produce menthol.*ester containing short chain fatty acid was used as a substrate for determination of esterase units. Amongst the 6 shortlisted isolates VM-3 and BG-1 found to be most potent esterase producers with highest esterase units 143 and 50.respectively. (Figure 1).

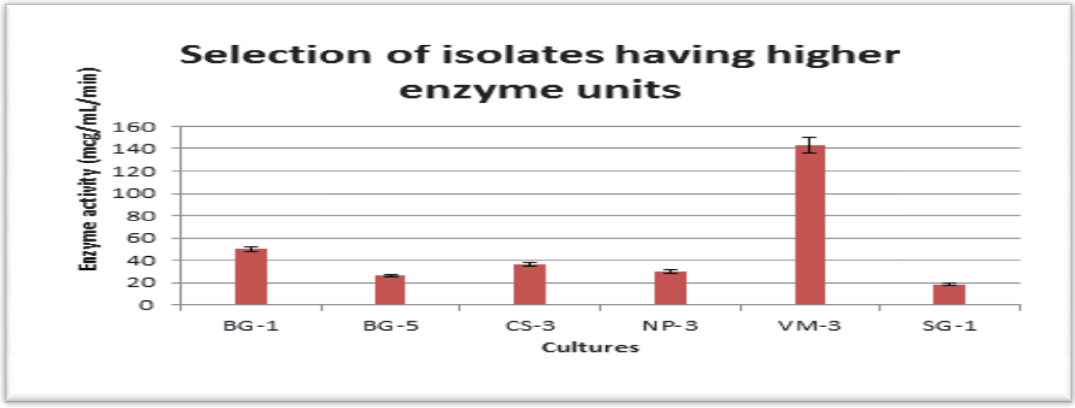


Figure 1. Esterase enzyme activity of shortlisted microbial strains

Reactions were carried out in 100 mM sodium phosphate buffer at 100 mM p-nitrophenyl butyrate concentration incubated at 37°C for 15 mins.

This was quite contradictory to the expected result as the strains that showed good activity ratio did not give the expected yield of esterase enzyme production when determined quantitatively. Thus based on the enzyme yields isolates VM-3 and BG-1 were selected for further studies.

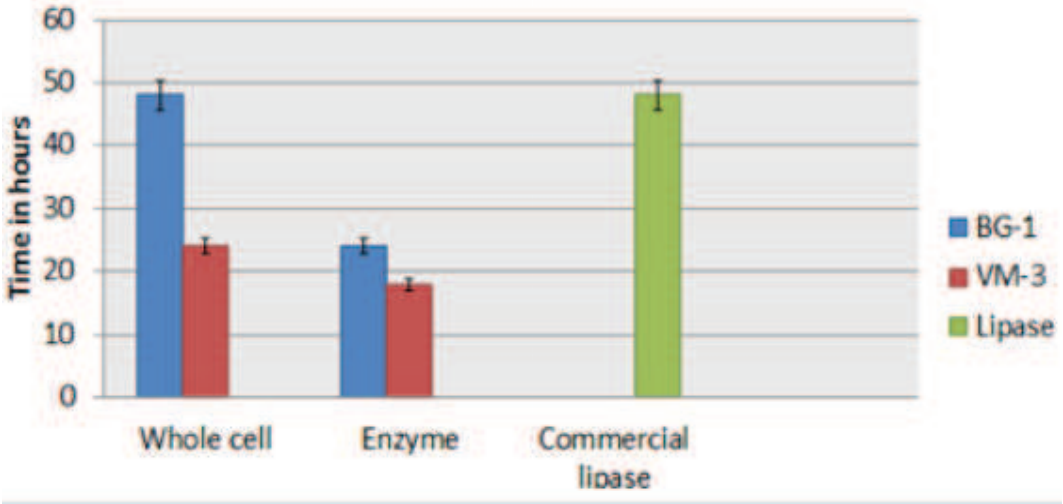
Hydrolysis of dl-menthyl acetate using

intracellular esterase enzyme and whole cells preparate as biocatalyst:

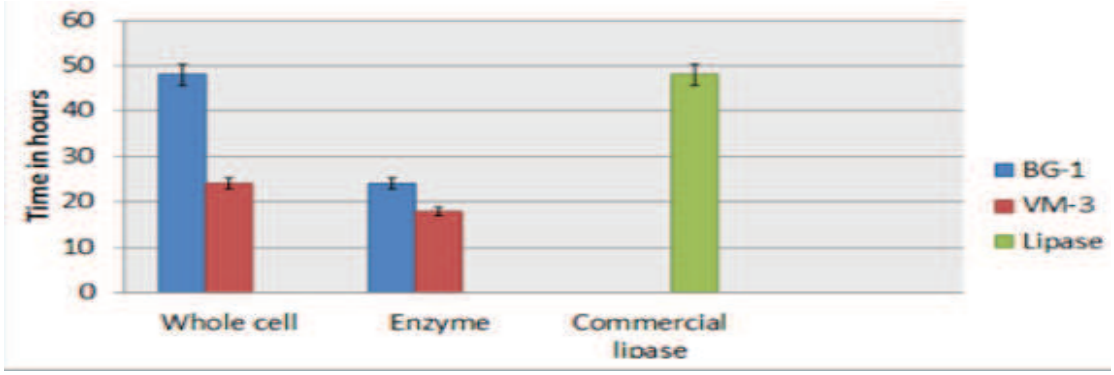
Compared to isolated enzymes, whole-cell biocatalyst has a number of attributes that are particularly attractive for large-scale applications [9] and has been widely adopted for the commercial synthesis of a variety of compounds, from bulk chemicals to valuable pharmaceuticals [11, 12]. The use of whole cells eliminates the need for tedious, expensive protein isolation and/or purification [13]. Additionally, with the protection of cell envelops and walls, enzymes are generally more stable in whole-cell systems. Hence, two shortlisted isolate's whole cell preparate as well respective enzymes were used for hydrolysis of *dl*-menthyl acetate to *l*-menthol and time period required for it was determined at a 100 mM concentration of substrate. Time period required for the hydrolysis of *dl*-menthyl acetate using enzyme preparate from both isolates gave fairly quicker product yield of menthol (Figure 2A) than that attained when whole cell biomass was used indicating that enzyme fractionates have better feasibility of use than that of whole cells in industry.

The kinetic resolution of *dl*-menthyl acetate catalyzed by commercial enzymes (Lipase D & Lipase PS) with equivalent activity was performed in order to compare their catalytic performance. Amongst these commercial enzymes Lipase D showed hydrolytic activity in 48 hrs whereas Lipase PS showed no hydrolysis activity in 48 hrs also , indicating that the enzyme preparation from procured strains were more promising biocatalyst than the tested commercial enzymes for industrial production of *l*-menthol.

Fig 2 : *l*-menthol production detected using 5% vanillin reagent



A. Using 100mM concentration of *dl*-menthyl acetate



B. Using 500mM concentration of *dl*-menthyl acetate

Additionally, use of high substrate/product concentration would be beneficial for practical application of an enzymatic process because it will reduce the cost of product isolation to a large extent. Thus the effect of the enzyme at higher concentration of substrate was studied. However, enzymatic activity is frequently inhibited by the existence of its own substrate at relatively high concentration. Thus, besides the hydrolytic activity and enantioselectivity, the substrate/ product tolerance of an enzyme is an important parameter to be evaluated for potential applications in industry and the effect of substrate concentration on the product formation was investigated at a fixed ratio of substrate to enzyme (S/E).

As shown in Figure 2B, time period required for hydrolysis of *dl*-menthyl acetate was affected due to the increase in substrate concentration to 500mM. As substrate concentration increased, time period required for menthol detection also increased by additional 24 hrs for whole cell preparate as well enzyme preparation. Commercial enzymes failed to catalyze hydrolysis of higher substrate concentration and *l*-menthol was not detected even after 48 hrs, indicating enzyme activity was inhibited by high substrate concentrations.

Determination of enatiomeric excess of the product: Enantiomeric excess of *l*-menthol formed was determined in order to measure its optical purity. Enzyme preparate of culture VM-3 was found to be stable and effective for *l*-menthol production at 100mM concentration and even at higher concentration of substrate (500mM) while, whole cells of VM-3 was unable to hydrolyze substrate even at a 100 mM concentration of substrate (Table 3). In contrast, enzyme preparate and whole cells of isolate BG-1 was able to hydrolyze substrate at 100 mM concentration but as the concentration of substrate was increased, hydrolytic activity of whole cells was inhibited.

Thus, enzyme preparate of isolate VM-3 exhibited the highest hydrolytic activity, with the best optical purity (100%) and strong tolerance against a high substrate concentration among the two shortlisted isolates. This was similar results obtained by Zheng et al (2009), who isolated strain ECU0554 from soil exhibiting the high hydrolytic activity, an optical purity (98%) and strong tolerance against a high substrate concentration (500 mM),. Yu et

Table 3. : Enantiomeric excess of l-menthol formed at 100 & 500 mM conc of *dl*-menthyl acetate determined by chiral GC analysis.

	BG-1				VM-3			
Substrate concentration	100 mM		500 mM		100 mM		500 mM	
	Whole cell	Enzyme	Whole cell	Enzyme	Whole cell	Enzyme	Whole cell	Enzyme
Time of incubation (Hrs)	48	24	48	48	24	18	48	24
Enantiomeric excess of l-menthol (%)	99.5	99.7	Not present	100	Not present	100	Not present	100

al (2007) have also isolated two bacterial isolates from soil, whose enzyme preparate having enantioselectivity greater than 95% towards *l*-menthol.

Summary and Conclusion:

Two bacterial strains, BG-1 and VM-3, which exhibited high hydrolytic activity and excellent enantioselectivity towards *l*-menthyl ester, were successfully isolated from soil samples through enrichment culture. The esterase extracted from BG-1 and VM-3 showed the best catalytic properties for *dl*-menthyl acetate. Enantioselective hydrolysis of 100 mM *dl* menthyl acetate at 30°C and pH 7.0, using crude enzymes as biocatalyst and 10% ethanol (v/v) as cosolvent, resulted in 100.0%ee for the *l*-menthol produced, which were much better than those using commercial enzymes tested. Moreover, it exhibited strong tolerance against high substrate concentration (up to 500 mM). These results imply that estereases enzymes can be potentially promising biocatalyst for the large-scale enzymatic

preparation of *l*-menthol. Using this excellent biocatalyst, the enzymatic production of *l*-menthol will become a mild, efficient, inexpensive and easy-to-use "green chemistry" methodology.

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Bhathena Z. P. and Revandkar Shital

Dept of Microbiology, Bhavan's College,

Andheri, Mumbai 400 058, India