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# Production, partial purification and characterization of organic solvent tolerant lipase from *Burkholderia multivorans* V2 and its application for ester synthesis

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ABSTRACT

*Burkholderia multivorans* V2 (BMV2) isolated from soil was found to produce an extracellular solvent tolerant lipase (6.477 U/mL). This lipase exhibited maximum stability in *n*-hexane retaining about 97.8% activity for 24 h. After performing statistical optimization of medium components for lipase production, a 2.2-fold (14 U/mL) enhancement in the lipase production was observed. The crude lipase from BMV2 was partially purified by ultrafiltration and gel permeation chromatography with 24.64-fold purification. The $K_m$ and $V_{max}$ values for partially purified BMV2 lipase were found to be 1.56 mM and 5.62 μmoles/mg min. The metal ions $Ca^{2+}$, $Mg^{2+}$ and $Mn^{2+}$ had stimulatory effect on lipase activity, whereas $Cu^{2+}$, $Fe^{2+}$ and $Zn^{2+}$ strongly inhibited the lipase activity. EDTA and PMSF at 10 mM concentration strongly inhibited the lipase activity. Non-ionic and anionic surfactants stimulated the lipase activity. BMV2 lipase was proved to be efficient in synthesis of ethyl butyrate ester under non-aqueous environment.



## 1. Introduction

Lipases (triacylglycerol hydrolases E.C.3.1.1.3) have proved to be better biocatalysts for performing various reactions such as esterification, transesterification, stereospecific hydrolysis of racemic esters and organic synthesis under water-restricted environment (Soni and Madamwar, 2000; Ferrera et al., 2005; Berglund and Hutt, 2000). Microbial lipases that can function as catalysts in non-aqueous solvents offer new possibilities such as shifting of the thermodynamic equilibria in favor of synthesis, enabling the use of hydrophobic substrates, controlling substrate specificity by solvent engineering and improving thermal stability of the enzymes (Koops et al., 1999). Apart from all the advantages, major limitation in carrying out the reaction under water-restricted environment is the tendency of organic solvents to strip water molecules from enzyme surface especially into the active site leaving the enzyme inactive (Yang et al., 2004). To overcome these limitations, several strategies like chemical modification of amino acids on enzyme surface (DeSantis and Jones, 1999), protein engineering (Magnusson et al., 2005), medium engineering (Laane, 1987), use of ionic liquids (Katalin et al., 2002) (supercritical fluids) and co-lyophilization with non-buffer salts (Mine et al., 2003) for enhancing enzyme activity and stability have been demonstrated. Alternately, it has been proposed that instead of modifying enzyme for increasing solvent stability, it would be more desirable to screen for naturally evolved solvent tolerant enzymes for application in non-aqueous enzymatic synthesis. There are many reports of purification and characterization of microbial lipases but very few reports are available on screening of lipase for its solvent tolerant ability (Rahman et al., 2006; Salameh and Wiegel, 2007; Nawani and Kaur, 2000).

Baharum et al. (2003) reported solvent tolerant lipase produced by *Pseudomonas* sp. strain S5 that was stable in organic solvents such as *n*-hexane, cyclohexane, toluene and 1-octanol. Rahman et al. (2006) also reported organic solvent tolerant lipase which was not only stable in *n*-hexane but its activity was stimulated in presence of *n*-hexane. Recently, lipases from *Burkholderia sp.* have attracted much attention due to its remarkable properties like substrate specificity, enantioselectivity (Weissfloch and Kazalauskas, 1995), thermal stability and solvent tolerance (Sugihara et al., 1992; Rathi et al., 2000, 2001) which makes it suitable for industrial use. Several authors have reported the purification, characterization and/or optimization of lipase production from *Burkholderia sp.* isolated from various sources (Park et al., 2007; Gupta et al., 2005). Based on the phylogenetic analysis of amino acid sequence, amongst the six groups, lipases from *Burkholderia sp.* have been classified in Group I.2, which possess more than 80% of amino acid sequence homology between the group members (Jaeger et al., 1999; Kim, 2003).

We have isolated a solvent tolerant lipase producing bacterium identified as *Burkholderia multivorans* V2 (BMV2). In this study, we report optimization of lipase production, partial purification, characterization and application of organic solvent tolerant lipase from

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*B. multivorans* V2 sp. for ester synthesis. The main highlight of our study is the application of this partially purified lipase in the synthesis of food ester under water-restricted environment.

## 2. Methods

Tributyrin oil and bovine serum albumin (protein standard) were obtained from HiMedia, India. Olive oil was procured from Figaro (Spain), para-nitro phenylpalmitate (*p*NPP) was purchased from Lancaster, France; gum arabic from Titan Biotech, India; bile salts and Bushnell Haas medium (BHM) (Himedia) India; isopropanol (Qualigens), India. All other solvents and chemicals used during the experiment were of analytical grade.

### 2.1. Bacterial strain

The bacterial culture used in this study was isolated by vigorous screening for organic solvent tolerant bacteria from oil contaminated soil samples collected from edible oil storage depots in Anand, Gujarat, India. The organic solvent tolerant organisms thus obtained were then subjected to further screening for potential lipase producer.

### 2.2. Identification of isolated bacterium

One bacterial strain designated as V2 was selected, which was able to produce organic solvent tolerant lipase; this culture as then identified as *B. multivorans* using GN2 plates and identification was done using MicroLog1 Identification system (Biolog Inc., USA). This was further confirmed following 16S rRNA gene sequencing. Genomic DNA of isolate was extracted as described by Ausubel et al. (1997). The genomic DNA was diluted properly to (20–50 ng) and used as template in PCR reaction (30 μL) using universal eubacteria primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) custom synthesized (BIORON, GmbH, Ludwigshafen, Germany). The amplification of 16S rRNA gene was done in BioRad PCR cycler (USA). Each PCR cycle (35 cycles in total) consisted of a 2 min denaturation step at 94 °C, followed by a 1 min annealing step at 55 °C, and a 1.5 min elongation step at 72 °C, with an initial denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 15 min. PCR products were resolved on a 1.2% (w/v) low-melting-point agarose gel in 1× TAE buffer, with a 1 kb ladder (BIORON, GmbH, Ludwigshafen, Germany) and visualized with ethidium bromide staining in Gel documentation (Alpha-Inotech, USA). The amplified PCR product was subjected to sequencing by automated DNA analyzer 3730 using ABI PRISM® BigDye™ cycle sequencing kit (Applied Biosystems, Foster City, CA). The nearly complete sequence (>99%) has been submitted to Genbank at NCBI. BLAST (*n*) program at NCBI server was used to identify and download nearest neighbour sequence from BLAST database (Altschul et al., 1997). The phylogenetic tree was constructed by neighbour joining algorithm using Kimura 2 parameter distance and more than 1000 replicates in Molecular Evolutionary Genetics Analysis 4.1 software (Kumar et al., 2001).

### 2.3. Organic solvent tolerance of BMV2 lipase

The BMV2 culture was streaked on tributyrin agar plates upon which each plate was flooded with 7 mL of different solvent (*n*-hexane, isooctane, benzene, ethyl acetate, toluene, cyclohexane, dimethyl fluoride and dimethyl sulfoxide). The stability of lipase in organic solvents was investigated by mixing 1 mL of culture supernatant (crude lipase) and 3 mL of solvent (*n*-hexane or isooctane) in screw cap test tubes and incubated in shaking water bath (50 rpm) at 37 °C. At varying periods of incubation one test-tube was removed and lipase activity was assayed in the aqueous phase by *p*NPP hydrolysis.

### 2.4. Profile of lipase production by BMV2

The 500 mL Erlenmeyer flasks containing 200 mL BHM amended with 1% (v/v) olive oil was inoculated with an overnight grown culture of BMV2 to obtain an initial culture density ($OD_{660\ nm}$) of 0.04–0.05 and incubated on orbital shaker (150 rpm) at 37 °C. The aliquots of 5 mL were withdrawn after every 24 h of interval and monitored for growth and lipase activity. The inoculum was prepared by transferring bacterial cells from a single isolated colony into a 5 mL sterile Luria broth using a sterile inoculating needle and incubated under shaking condition (150 rpm, orbital shaker) at 37 °C for 16–18 h.

### 2.5. Effect of different oils as carbon source on lipase production

The lipase production by BMV2 was studied employing BHM containing different carbon sources (olive oil, corn oil, groundnut oil and castor oil) at a concentration of 1% (v/v). The 250 mL flasks containing 100 mL of respective media were inoculated with overnight grown culture and incubated at 37 °C for 5 days under shaking condition (150 rpm). After 5 days of incubation, the lipase activity was monitored in culture supernatant obtained by centrifugation of culture broth at 12,857g for 20 min at 4 °C.

### 2.6. Media optimization for lipase production using response surface methodology

Since conventional method (one factor at a time) used for optimization does not indicate the interactions between the significant components, statistical approach for optimizing the significant media components has been employed. The experiments were performed using face centered central composite design using the statistical software package Design Expert 7.0, Stat-Eas Inc., Minneapolis, USA. Prior to RSM, Plackett-Burman design was used to screen the factors that influence the lipase production and those found significant based on studentized *T*-test were analyzed using RSM eliminating the insignificant ones. The *p* value (significance level) for the components olive oil, Trition X-100 and time were found to be less than 0.05 and hence selected for further optimization by RSM.

The quadratic model with 3 variables is represented as:

$$Y = 11.49 + 2.56 * A + 0.15 * B + 1.80 * C - 0.12 * A * B + 2.16 * A * C - 0.37 * B * C - 2.86 * A^2 - 2.43 * B^2 - 1.92 * C^2$$

where Y is lipase activity in cell free supernatant at the end of fermentation and *A* (Triton X-100), *B* (olive oil), *C* (time of incubation) are coded forms of the test variables. The minimum and maximum ranges of variables are coded as −1 and +1, whereas the central coded value is coded as 0. The statistical analysis was performed using the analysis of variance (ANOVA). The design of the experiment employed is represented in Table 1.

### 2.7. Partial purification of BMV2 lipase

The culture supernatant containing extracellular lipase obtained from a 5 day fermented broth of BMV2 was treated with 0.4 M $CaCl_2$ in order to precipitate fatty acids followed by centrifugation at 4 °C and 12,857g for 30 min. The supernatant was collected in a glass beaker and to it chilled acetone was added slowly, with continuous stirring, up to 70% (v/v) concentration and kept at −20 °C for 4 h to allow protein precipitation. The precipitates were then harvested by centrifugation at 4 °C and 12,857g