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Biochemical and biotechnological studies on a novel purified *bacillus* cholesterol oxidase tolerant to solvent and thermal stress

Fathy N ElBaza, Rawia F Gamal, Ashraf F ElBaza, Nasser E Ibrahim and Ahmed ElMekawy

Department of Industrial Biotechnology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City (USC), Sadat City, Egypt; Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Cairo, Egypt; Department of Bioinformatics, Genetic Engineering and Biotechnology Research Institute, University of Sadat City (USC), Sadat City, Egypt; Faculty of Engineering, Computer and Mathematical Sciences, School of Chemical Engineering, University of Adelaide, Adelaide, Australia

ABSTRACT

A novel bacterial strain was isolated and identified as *Bacillus pumilus*, with the capability to produce cholesterol oxidase enzyme (55 kDa). The production of the enzyme was optimized via a two-step statistical approach. Out of eight factors screened in Plackett–Burman, only four had significant effects on enzyme activity. The optimization process of these four variables by Box–Behnken revealed that the maximum enzyme activity (90 U/mL) was significantly obtained after 6 days of fermentation with 0.3%, 1% and 0.2% of NH4NO3, yeast extract and Tween 80, respectively. The purified enzyme showed optimum activity at pH 7.5 and temperature of 40 °C. The enzyme retained 100% of its activity after storage at 40 °C for 60 min. The enzyme also exhibited enhanced stability in the presence of Tween 80, methanol and isopropanol. This solvent and thermal stress tolerant enzyme, produced by *B. pumilus*, may provide a practical option for industrial and analytical applications.

Introduction

Cholesterol (5-cholesten-3-ol) is a type of steroid, mainly located in the membranes of animal cells (Hassanein et al. 2012). It has been related to colon cancer development and the strong toxic effects of a number of cholesterol oxides suggested a possible role for them in the occurrence of cardiovascular diseases, attributed to the high levels of blood cholesterol (Kim et al. 2002). Due to the importance of monitoring the two types of cholesterol (low-density lipoprotein (LDL) and high-density lipoprotein (HDL)) in serum for the diagnosis of atherosclerotic or hyperlipaemia diseases, some methods have been developed for the separation of HDL and LDL cholesterols using different types of detergents (Praveen et al. 2011). These efforts focused on developing sensitive *in vitro* analysing methods for monitoring and measuring cholesterol in different biological samples (Saxena and Goswami 2010; Saxena et al. 2011). The enzymatic technique, utilizing cholesterol oxidase (ChO; EC 1.1.3.6) enzyme, is a common method among cholesterol assessment techniques (Kasabe et al. 2015). Some bacterial species are capable of degrading cholesterol by ChO, so that cholesterol is oxidized to 4-cholesten-3-one, and oxygen is reduced to hydrogen peroxide as shown in Figure 1 (Kim et al. 2002).

This enzyme has a wide range of clinical and industrial applications, for example, food, agricultural and pharmaceutical fields, leading to a considerable increased demand for it (Niwas et al. 2013). Several bacterial genera can produce ChO with the ability to assimilate cholesterol as a single carbon source, such as *Streptomyces* (Praveen et al. 2011; Niwas et al. 2013), *Rhodococcus* (Yazdi et al. 2008; Ahmad and Goswami 2014; Kasabe et al. 2015), *Bardetella* (Lin et al. 2010), *Chromobacterium* (Doukyu et al. 2008), *Enterobacter* (Ye et al. 2008), *Pseudomonas* (Ghosh and Khare 2016), *Mycobacterium* (Yao et al. 2013) and *Bifidobacterium* (Park et al. 2008). Stable and sensitive ChO is a current crucial need in order to get the benefit of this multipurpose enzyme. These needs might be achieved by enhancing the current ChO producer strains by employing the contemporary recombinant biotechnologies, although they entail some drawbacks like the development of inclusion bodies, heterologous protein degradation and improper protein folding.
An alternative approach is to select some novel microbial producers with the ability to produce ChO with superior stability and stress tolerance for improved applicability, for example, to enhance the differential assay technique for measuring LDL and HDL cholesterols in serum.

The objective of our study is the identification and isolation of new Bacillus strains, from the oily waste of ships that have the potential to produce tolerant and stable ChO under different conditions in order to be applied in a wide range of medical fields. In the course of the study, the taxonomic classification of a novel enzyme producer bacterial strain is described. Moreover, the fermentative conditions of the enzyme production are statistically optimized through a two-step approach using Plackett-Burman and Box-Behnken experimental designs. The enzyme is purified and its pH tolerance, thermal stability and solvents tolerance are assessed.

Materials and methods

Materials and samples

The oily waste samples were kindly provided by Department of Industrial Biotechnology in University of Sadat City located in Egypt. Cholesterol and 4-cholesten-3-one were purchased from Sigma Chemicals (St. Louis, MO). Yeast extract and agar were supplied by Oxoid (Hampshire, UK). Gel filtration column and Sephadex G-100 were purchased from Pharmacia, Uppsala, Sweden. Protein marker was brought from Affymetrix USB (Cleveland, OH). Bradford protein assay kit was purchased from Bio-Rad Laboratories, Richmond, CA. All other chemical reagents, solvents and salts were of analytical grade and purchased from local suppliers.

Isolation and ChO screening of bacterial cells

The samples (5% v/v) were inoculated into cholesterol enrichment medium (1 g NH₄NO₃, 0.25 g K₂HPO₄, 0.25 g MgSO₄, 0.001 g FeSO₄, 5 g yeast extract, 1 g cholesterol (dissolved in 0.1% Tween 80) and distilled water up to 1 L; pH 7) and incubated under shaking condition at 37°C for 7 days. Enriched cultures were serially diluted with saline solution and plated on modified cholesterol agar (Praveen et al. 2011). Colonies were examined for halos and positive ones were isolated by frequent single-colony isolation on the same medium. Pure colonies were streaked on ChO indicator agar medium plates (1 g cholesterol, 1 g Triton X-100, 0.1 g o-dianisidine, 1000 U of peroxidase, 15 g agar and distilled water up to 1 L) and incubated for 2–4 days at 37°C (Niwas et al. 2013). The growing colonies were examined for the formation of brown pigments surrounding them and positive ones were isolated as ChO-producing bacteria and maintained at 4°C for further study. The ChO production by different isolates was verified in Erlenmeyer flasks (250 mL), each containing 100 mL modified cholesterol growth medium. The flasks were inoculated with 1% of cells suspension and incubated at 37°C on a rotary shaker (150 rpm) for 7 days, after which the enzyme activity was assayed.

Taxonomic studies of the ChO producer strain

The highest ChO-producing bacterial culture was determined and identified through 16S rRNA homology technique. Isolation of DNA, amplification by polymerase chain reaction (PCR), and sequencing of the amplified product was performed according to the earlier reported procedures (Praveen and Tripathi 2009). The pairwise sequence alignment was carried out via BLAST (http://www.ncbi.nlm.nih.gov/) tool and multiple sequence alignment by CLUSTALW. The phylogenetic tree was created by neighbor-joining approaches using MEGA 7 software (State College, PA, USA). The resulting nucleotide sequence was deposited in the GenBank database under accession number KX685666.
**ChO assay and protein determination**

The activities of extracellular and membrane-bound enzymes were measured according to the technique of Inouye et al. (Yehia et al. 2015). The total assay mixture was 0.525 mL, containing 0.1 mL of enzyme solution in 0.4 mL Tris-HCl buffer (125 mM, pH 7.5), incubated for 3 min at 37 °C, and 25 μL of cholesterol (12 mM) dissolved in isopropanol. Absolute ethanol (2.5 mL) was added after 30 min, and the 4-cholesten-3-one degradation product amount was determined by measuring its absorbance at 240 nm. Authentic 4-cholesten-3-one was used as analytical comparative standard (10–100 μg/mL dissolved in isopropanol). One unit of the ChO activity was defined as the amount of enzyme which releases 1 μmol of 4-cholesten-3-one from cholesterol per minute. The total proteins were assessed using Bradford’s method applying bovine serum albumin (BSA) as the standard (Bradford 1976).

**Fermentative optimization of ChO production**

The production of ChO enzyme was optimized employing modified cholesterol medium with different examined variables, with ChO activity measured as the dependent response. Eight variables were screened, via the application of Plackett–Burman design (PBD), based on the impact percentage of each variable (Abdel-Monem et al. 2012; ElBaz et al. 2016). The tested variables include cholesterol concentration, pH, harvesting time, NH₄NO₃ concentration, yeast extract concentration, aeration (medium volume/flask volume), cholesterol addition time and Tween 80 concentration. Two levels were assigned for each variable; low (−1) and high (+1) levels (Table 1). The matrix of the tested variables resulted in 27 experiments. To predict the optimum point, a second-order polynomial equation was fitted for the correlation of the relationship between the ChO activity and the independent variables (ElMekawy et al. 2013).

**Purification of the ChO enzyme**

All the purification steps were performed at 5 °C. The growing culture broth was centrifuged at 10⁶×g for 10 min and the cell-free supernatant was exploited as an extracellular crude ChO source. To obtain the membrane-bound enzyme, the collected cells were washed twice using ethyl acetate and then frozen at −18 °C. The extraction of membrane-bound ChO from frozen cells was carried out in 1 mM phosphate buffer (pH 7), containing 0.7% Triton X-100 (v/v), for 18 h at 5 °C under continuous stirring. The extracted enzyme was subsequently centrifuged and the cell-free supernatant was used as a crude membrane-bound enzyme source.

The ChO enzyme precipitation by ammonium sulphate was optimized, using different saturation concentrations (45, 50, 60, 70 and 75%) under different pH values (5.5, 6, 7, 8 and 8.5), through 10 trials. Ammonium sulphate was gradually added to the crude ChO solution with continuous stirring till it was completely solubilized at the defined pH value. The ammonium sulphate traces in the precipitated pellet were removed by dialysis against distilled water in a cellophane bag (MWCO 10 kDa) for 3 h, followed by dialysis against phosphate buffer (pH 7.5). The obtained concentrate was then completely loaded in a Sephadex G-100 gel filtration column with dimensions of 2 cm × 60 cm. The column was equilibrated and eluted with 0.1 M phosphate buffer (pH 7.5) at 20 mL/h. Three millilitres from each fraction was sampled

**Table 1. Independent variables and their verified levels for the Plackett–Burman and Box–Behnken experimental designs.**

<table>
<thead>
<tr>
<th>Design type</th>
<th>Independent variable</th>
<th>Symbol</th>
<th>Actual values</th>
<th>Coded levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBD</td>
<td>Cholesterol conc. (w/v %)</td>
<td>X₁</td>
<td>0.05</td>
<td>−1</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>X₂</td>
<td>5.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Harvesting time (days)</td>
<td>X₃</td>
<td>6</td>
<td>−1</td>
</tr>
<tr>
<td></td>
<td>NH₄NO₃ conc. (w/v %)</td>
<td>X₄</td>
<td>0.05</td>
<td>−1</td>
</tr>
<tr>
<td></td>
<td>Yeast extract conc. (w/v %)</td>
<td>X₅</td>
<td>0.3</td>
<td>−1</td>
</tr>
<tr>
<td></td>
<td>Aeration</td>
<td>X₆</td>
<td>0.15</td>
<td>−1</td>
</tr>
<tr>
<td></td>
<td>Cholesterol addition time (day)</td>
<td>X₇</td>
<td>0</td>
<td>−1</td>
</tr>
<tr>
<td></td>
<td>Tween 80 conc. (v/v %)</td>
<td>X₈</td>
<td>0.4</td>
<td>−1</td>
</tr>
<tr>
<td>BBD</td>
<td>Harvesting time (days)</td>
<td>X₁</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>NH₄NO₃ conc. (w/v %)</td>
<td>X₄</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Yeast extract conc. (w/v %)</td>
<td>X₅</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Tween 80 conc. (v/v %)</td>
<td>X₈</td>
<td>0.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>
and analysed for protein content and Cho activity. The purified Cho was assessed for the existence of its FAD cofactor by measuring its absorption at 360 and 450 nm to determine the presence of FADH$_2$ (reduced state) and FAD (oxidized state), respectively (Pandini et al. 2010).

**Enzyme molecular weight determination**

The molecular weight of the purified enzyme was determined by Na dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) in a Mini Protean II vertical tank apparatus (Bio-Rad, Munich, Germany) applying acrylamide (10%) separating gel along with acrylamide (5%) stacking gel including 0.1% SDS. A mixture of the purified enzyme and reducing buffer was prepared, then heated at 90°C for 4 min, and introduced into separate lanes. Standard proteins with medium molecular weight range were loaded onto the first lane and the gel was stained with Coomassie Brilliant Blue R-250.

**Biochemical characterization of purified Cho**

**Effect of pH on Cho activity and stability**

The optimum pH of the Cho activity was determined by measuring absorbance at 240 nm, attributable to the formation of 4-cholesten-3-one, and the residual enzyme activity was then calculated. The optimum enzyme activity was detected within a pH range of 5–8.5 at 37°C for 5 min via 100 mM sodium acetate buffer (pH 5–5.5), 100 mM phosphate buffer (pH 6–7.5), 50 mM Tris-HCl buffer (pH 8) and 100 mM glycine buffer (8.5). The pH stability of Cho was also measured after the enzyme was incubated at 37°C for 60 min in buffers with different pH values (6.5, 7.5 or 8.5) and the residual enzyme activity was determined every 10 min.

**Effect of temperature on Cho activity and stability**

The optimum temperature for Cho enzyme activity was verified, using 100 mM phosphate buffer (pH 7.5), through its exposure to a range of different temperatures from 20–70°C with increments of 5°C. Furthermore, the thermal stability was examined by incubating the Cho enzyme at pH 7.5 at a temperature range of 40–60°C for 0–60 min with increment of 10 min. The effect of the enzyme storage in refrigerator (4°C) on its stability was also studied. The Cho enzyme, in 0.1 M phosphate buffer (pH 7), was stored for 30 days in refrigerator and the residual activity was assayed every five days. The effect of temperature on enzymatic reaction was expressed in terms of the temperature coefficient Q$_{10}$, which is a measurement for the reaction velocity upon increasing the temperature by 10°C.

**Effect of different cholesterol solubilizers**

Different solvents are often used as solubilizers of cholesterol, for that reason the stability of Cho enzyme was examined in the presence of various solubilizers. Cholesterol (0.1 g) was completely dissolved at 60°C in 1 mL of methanol, ethanol, isopropanol or Tween 80 and the dissolved cholesterol was added to 1 mL of enzyme solution. A combination of two solubilizers; methanol and Tween 80, was also examined via two methods. In the first method, cholesterol (0.1 g) was completely dissolved at 60°C in 1 mL methanol followed by the addition of Tween 80 (0.4 mL), while in the second one, cholesterol was completely dissolved at 60°C in Tween 80 followed by the addition of methanol using the same amounts as the first method. The relative activities were measured after the incubation with all solubilizers.

**Results and discussion**

**Isolation and identification of Cho-producing bacterium**

Bacterial colonies were isolated from the oily samples to be screened for their capability to produce Cho enzyme. After the isolation of colonies, 20 bacterial isolates formed halos around their growing colonies on the agar medium, showing the ability to grow on cholesterol medium with cholesterol as a single-carbon source. Cholesterol is converted to 4-cholesten-3-one and hydrogen peroxide by Cho, resulting in the formation of azo compound and the colour of the Cho indicator medium becomes dark brown. Out of 20 bacterial isolates, only 16 were able to form brown pigment around them. Quantitative determination of the Cho activity of the selected isolates was performed through fermentation in cholesterol medium. The bacterial strain with highest Cho activity (16.8 U/mL) was selected for advanced studies. The selected isolate was identified with 16S rRNA homology analysis. The phylogenetic tree was obtained by means of some neighbours that possess the maximum homologous gene sequences (Figure S1). Sequence of the isolated strain, with highest Cho activity, was highly similar (98%) to the 16S rDNA sequence of *Bacillus pumilus* strain MS5-14 (GenBank accession no. KX685666).
The *B. pumilus* ChO had not been investigated so far, and as a result, its characteristics were further studied.

**ChO production optimization**

The PBD was statistically employed for the screening of different variables that are significantly affecting the ChO production by *B. pumilus* (Table S1). The ChO activity values widely ranged from 0.06 to 9 U/mL, which highlights the importance of screening the significant effects of growth conditions and medium components on the enzyme activity (Elbaz et al. 2015). The lowest ChO activity was noticed in the 5th run, while the highest one was obtained in the 1st run. This improvement in the enzyme activity was obtained under pH 8 after 5 days, with medium/flask ratio of 0.35 and the concentrations of cholesterol, NH₄NO₃, yeast extract and Tween 80 were 0.2, 0.05, 0.3 and 0.4%, respectively.

These significant factors were further optimized via BBD which involves regression equation, connecting the response to the coded levels of the independent variables. The significant variables, namely the harvesting time (*X₃*), NH₄NO₃ (*X₄*), yeast extract (*X₅*) and Tween 80 (*X₆*) concentrations, were matrixed in the BBD with three levels for each of them. The non-significant variables were stabilized at their low (−1)/high (+1) levels depending on their negative/positive effect values, respectively. The results showed that harvesting time, yeast extract and Tween 80 concentrations significantly increased the *B. pumilus* ChO activity (Table S2). The maximum ChO activity (90 U/mL) was obtained in the 8th run after 6 days upon using concentrations of 0.3, 1 and 0.2% for NH₄NO₃, yeast extract and Tween 80, respectively. This activity was tenfold more than that obtained from the PBD screening experiment.

The amount and type of carbon and nitrogen substrates have been the focus of several industrial investigations in order to obtain cost-effective growth media components. This type of research is particularly important when the optimum growth conditions of the applied microbial strains are unknown. These nutritional factors, in addition to growth conditions, for example, harvesting time, pH and aeration, have been noticed to determine the optimum growth and the ability of bacterial cells to produce cellular products (Tari et al. 2007). Accordingly, one aim of this study was to optimize growth medium components using different concentrations of NH₄NO₃, yeast extract and Tween 80, resulting in maximum ChO production of *B. pumilus* and recognizing the other essential factors that would also bring about high ChO production.

Generally, the bacterial ChO production has been optimized in very few studies (Moradpour et al. 2014). After comparing the current results with the previously conducted studies on ChO production from various bacterial isolates, it was evidently observed that *B. pumilus* enzyme activity surpassed those results. In two studies performed to optimize the ChO production by *Streptomyces* sp. using different carbon/nitrogen sources and cholesterol inducer concentration, the highest activity obtained was 6 U/mL and 20 U/mg (Praveen et al. 2011; Niwas et al. 2013). Similarly, the *Bordetella* ChO production was optimized, and the maximum activity was 1.7 U/mL (Lin et al. 2010). Moreover, the ChO produced by *Enterobacter* and *Rhodococcus*, without optimization, showed activity of 0.43 and 0.398 U/mL, respectively (Ye et al. 2008; Kasabe et al. 2015).

**Purification and identification of bacterial ChO**

The purification of the crude ChO was performed through two consecutive stages, starting with ammonium sulphate precipitation, then gel filtration chromatography using Sephadex G-100 column. The ChO precipitation was optimized using different concentrations of ammonium sulphate under different pH values. The optimum conditions for the precipitation process was 60% saturation with ammonium sulphate under pH 7, which resulted in the highest specific activity (0.14 U/mg protein) (Table 2). After applying these conditions, the fraction precipitated with ammonium sulphate displayed a total enzyme activity of 17 U, corresponding to 68% of the recovered activity with a total protein amount of 118 mg. Although 45 fractions were collected from the Sephadex G-100 column, the maximum enzymatic activity was only detected in fractions 28–35 (Figure 2). Being a flavo-protein, ChO shows a maximum absorption at 450 and 360 nm in its oxidized and reduced states, respectively (Kasabe et al. 2015). The majority of the collected fractions (40) showed absorptions at 360 and 450 nm, but

- Table 2. Optimization of the first step purification of ChO precipitation by ammonium sulphate.

<table>
<thead>
<tr>
<th>Ammonium sulphate conc. (%)</th>
<th>pH</th>
<th>ChO (U/mL)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>5.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>7.0</td>
<td>33</td>
<td>0.14</td>
</tr>
<tr>
<td>45</td>
<td>5.5</td>
<td>12</td>
<td>0.02</td>
</tr>
<tr>
<td>60</td>
<td>7.0</td>
<td>31</td>
<td>0.13</td>
</tr>
<tr>
<td>60</td>
<td>8.0</td>
<td>15</td>
<td>0.03</td>
</tr>
<tr>
<td>45</td>
<td>8.5</td>
<td>3</td>
<td>0.007</td>
</tr>
<tr>
<td>60</td>
<td>6.0</td>
<td>43</td>
<td>0.1</td>
</tr>
<tr>
<td>70</td>
<td>7.0</td>
<td>29</td>
<td>0.05</td>
</tr>
<tr>
<td>50</td>
<td>7.0</td>
<td>19</td>
<td>0.09</td>
</tr>
<tr>
<td>75</td>
<td>8.5</td>
<td>8</td>
<td>0.01</td>
</tr>
</tbody>
</table>
80% of these fractions had shown very low ChO activity. Eight fractions exhibiting the maximum recovered activity, were pooled leading to a total ChO activity of 4.8 U and specific activity of 1.37 U/mg. The purification process totally resulted in an escalation in specific activity from 0.13 U/mg in crude broth to 1.37 U/mg after the gel filtration chromatography step (Table 3).

The molecular weight of the purified ChO, according to SDS-PAGE analysis, was 55 kDa in the form of one protein band stained on the gel, which emphasizes the purified enzyme homogeneity (Figure 3). A number of studies investigated the production and purification of ChO from different bacterial isolates. They reported a molecular weight in the range of 55–62 kDa (Praveen et al. 2011, Niwas et al. 2013), which coincided with the molecular weight of the B. pumilus purified ChO enzyme.

**pH stability**

Generally, every enzyme is active in a certain range of pH, and hence, a particular optimum pH has to be detected. The effect of different pH values on the activity and stability of the purified ChO are shown in Figure 4. After the examination of the effect of a pH range of 5–8.5 on the enzyme activity, the optimum pH was observed to be 7.5 (Figure 4(A)). The enzyme considerably lost its activity at a pH value less than 6.5. On the other side, the enzyme retained more than 80% of its activity at pH values of 7–8. The pH stability
was also examined by incubating the enzyme under pH values of 6.5, 7.5 or 8.5 for 60 min (Figure 4(B)). The enzyme reserved more than 80% of its activity after being incubated under tested pH values for 60 min. Nevertheless, only 10% of the enzyme activity vanished after 30 min under all pH values. These results revealed the high stability of the enzyme at pH range of 6.5–8.5. Comparable results were obtained with ChOs purified from other bacterial species, which kept most of their activities within pH range from 7 (Doukyu et al. 2008; Ye et al. 2008; Lin et al. 2010; Praveen et al. 2011; Niwas et al. 2013) to 8 (Kasabe et al. 2015). The role of pH is well known in changing the ionization status of the enzyme substrate complex, which affects the ionic bonds that help to determine the 3D structure of the enzyme, leading to enzyme inactivation (Illanes 2008).

**Thermal stability**

The thermal stability of the ChO enzyme has been well verified in this study. The ChO enzyme activity was examined under a temperature range of 20–70 °C. The optimum temperature was observed to be 40 °C, while the enzyme activity sharply declined at temperatures higher than 45 °C (Figure 5(A)). Moreover, the enzyme activity after incubation at various temperatures for 60 min was evaluated. The enzyme sustained 100% of its activity after 60 min when stored at 40 °C, whereas the same pattern of activity was observed when the enzyme was incubated at 50 °C with slight decline after 30 min till it reached 83% by the end of the incubation period (Figure 5(B)). On the other side, when the enzyme was incubated at 60 °C, its activity sharply declined till it reached 13% after 40 min, and the enzyme was totally inactivated by the end of incubation period. The enzyme stability was further checked at a lower temperature (4 °C) for elongated period of incubation (30 days). About 20% reduction in the activity was observed after 10 days, then the activity gradually declined until it reached 40% after 30 days (Figure 5(C)). The effect of temperature on the enzymatic reaction was determined in terms of the temperature coefficient (Q₁₀). The maximum obtained Q₁₀ (2.13) was observed when the temperature was raised from 30 °C to 40 °C (Figure 5(D)), which is in agreement with the results of the optimum temperature value for the enzyme activity and stability.
The optimum temperature (40°C) was within the range of the optimum temperature of the related bacterial ChO enzymes, which had values in the range of 25–65°C (Doukyu et al. 2008; Ye et al. 2008).

**Tolerance to solubilizers**

Organic solvents and detergents are regularly used to solubilize cholesterol. Stability of ChO was inspected in the presence of different organic solvents and detergent (Figure 6). ChO displayed an outstanding stability when solubilized in several organic solvents and detergent. The highest enzyme activity was obtained when methanol was used as a solvent (265%). Furthermore, the enzyme activity was enhanced (>100%) with all tested solubilizers except ethanol. The effect of the two solubilizers with highest enzyme activity (methanol and Tween 80) were further studied when combined with each other via two different methods. Solubilizing cholesterol in hot methanol (60°C) followed by the addition of Tween 80 was observed to be a suitable solvating and emulsifying method for higher ChO activity, which accounts for approximately 70% improvement compared to that of each single solubilizer.

The effect of different solvents and detergents on several bacterial ChO enzymes has been investigated in several studies. *B. pumilus* ChO activity was compared to other studies which applied the same solvents and detergents (Figure 7). The relative activities of different purified bacterial enzymes greatly diverged in the range of 4–250%. The minimum relative activity (4%) was obtained when the *Bordetella* ChO was subjected to methanol (Lin et al. 2010), while the maximum one (250%) was obtained upon solubilizing of *Streptomyces parvus* ChO in isopropanol (Praveen et al. 2011). It was clear that methanol exhibited an unprecedented qualitative and quantitative enhancement in the relative activity of the *B. pumilus* ChO, which accounts for more than twofold, compared to the maximum improvement of enzyme activity (115%) ever obtained in previous studies (Niwas et al. 2013). This enhancement also extended to Tween 80, which displayed a resultant relative activity (171%) superior to the maximum activity obtained after its application with *Streptomyces parvus* purified enzyme (150%) (Praveen et al. 2011). Furthermore, the application of isopropanol solvent with *B. pumilus* ChO resulted in an intermediate relative activity (131%) compared to other studies that gave effects ranging from 115% to 250% (Praveen et al. 2011; Kasabe et al. 2015). Accordingly, ChO from the isolated strain has shown improved stability in the presence of Tween 80, methanol and isopropanol.

Moreover, commercial enzymes from *Nocardia* sp., *Pseudomonas* sp., *Streptomyces* sp. and *Cellulomonas* sp. were reported to be inactivated upon the employment of methanol, ethanol and isopropanol (Niwas et al. 2013). The *B. pumilus* ChO enzyme under study was more stable in detergent/organic solvents compared to commercial ones and therefore can be applied in different chemical reactions, for example, bioconversion of several 3β-hydroxysteroids and synthesis of steroid hormones (Niwas et al. 2013). It has been known that alcohols generally create structural variations in proteins and peptides. Methanol improved the enzymatic activity possibly by acting as a molecular lubricant that enhances the enzyme’s...
conformational flexibility to facilitate its catalytic activity (Wiggers et al. 2007).

Conclusions

The purification and optimization of growth conditions and medium components for the B. pumilus ChO enzyme were exclusively performed in this study. Besides detecting the optimum growth conditions of this bacterial isolate, this study also worked as a paradigm for the application of statistical approaches with the bacterial systems to give the operator enough suppleness to select the optimum factors in terms of the enzyme production response via the polynomial model. After two sequential steps of optimization course, the highest ChO enzyme activity was obtained after 6 days upon the usage of NH₄NO₃ as a nitrogen source, at 0.3%. Moreover, the purification of B. pumilus ChO was performed by gel filtration chromatography and confirmed by using SDS-PAGE analysis in which single band was obtained and the enzyme molecular weight was determined to be 55 kDa. The purified bacterial ChO had thermal stability and tolerance for organic solvents, which are considered as beneficial features, rendering the newly isolated strain, B. pumilus, as a potential source of ChO which could be employed in clinical and various research purposes.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References


