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Stable release of enhanced organic solvent tolerant amylase from *Bacillus amyloliquefaciens* AMY02 under sub-merged fermentation

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This study has been performed to isolate a potential strain able to release the prolific amylase under non-aqueous conditions to meet the current demand in industries to substitute the amylase produced in aqueous media. A bacterial strain that produces organic solvent-stable amylase in the media containing 15% benzene was isolated from the soil. The recovered strain was identified to be *Bacillus amyloliquefaciens* AMY02by 16S rRNA sequencing. Under sub-merged fermentation, the optimized amylase release by this strain was found with the condition having starch (carbon source), pH 7.0, the temperature at 30°C for 48 h (incubation time). This optimized condition promoted the amylase production to be 2.04-fold higher than the culture was kept under standard condition with the basic media composition. Further, the stability of the enzyme in the presence of 20% organic solvents was assessed by incubating for 2 weeks. The enzyme was found to be active and stable in the presence of benzene, chloroform, o-xylene, and toluene. The higher organic solvent stability of this amylase production by *B. amyloliquefaciens* under sub-merged fermentation can be an alternative catalyst in non-aqueous media for industrial applications.

Keywords: Amylase production, Non-aqueous media, Phylogenetic analysis, Solvent-stable enzymes, Solvent tolerant bacteria

Generally, organic solvents are toxic to microorganisms and cause the cell lysis as well as disturbances in the structural and functional integrity of the cell[1-2]. However, some bacteria have undergone various adaptations such as the development of solvent efflux pumps, rapid membrane repair mechanisms, lower cell membrane permeability, increased membrane rigidity, and decreased cell surface hydrophobicity; these adaptations enable them to grow in the presence of organic solvents[3].Organic solvent tolerance is a strain-specific property and the toxicity of a solvent is correlated to the logarithm of its partition coefficient in *n*-octanol and water [log P_{ow}][4]. There are several advantages of synthesizing enzymes in organic solvents or aqueous solutions containing organic solvents. These include, the increased solubility of non-polar substrates, suppression of water-dependent side reactions, and the elimination of microbial contamination in the reaction mixture[5,6]. However, most of the enzymes are not stable, or partially stable in the presence of organic solvents. Various methods such as chemical modification, immobilization, entrapment, protein engineering, mutagenesis, and directed evolution have been used to increase the activity and stability of enzymes, in the presence of organic solvents[5,7]. However, naturally, solvent-stable enzymes with higher activity in the presence of organic solvents are preferred for industrial and other down-stream applications.

α-Amylase that catalyse the hydrolysis of α-1, 4-glycosidic linkages of starch into maltose, is one of the most important industrial enzymes. It has several applications in food, textile, detergent, baking, paper, starch processing, and pharmaceutical industries[8,9]. Additionally, this amylase is also used in the preparation of glucose syrup, baking, and brewing[10]; it accounts for approximately 25–30% of the total enzyme sales around the world. Amylases have been produced from several sources, such as plants, animals and microorganisms. However, microorganisms are widely represented as alternative sources for enzymes, owing to their ability to be cultured in large quantities with a short span of fermentation time, in addition they are easy to handle,

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cost-effectiveness and can be subjected to gene manipulation[11]. Among the wide range of microbial species used to produce amylases, bacterial source of amylase production is cheaper and faster than from other microbes. Because of bacterial positive attitude, the bacterial strains have been selected to produce large quantities of amylase to overcome the industrial demands.

Several researchers have reported the production of organic solvent-stable proteases and lipases from bacterial strains[12-20]. The α-amylase-producing strains were also isolated and characterized from various bacterial species[21-26], but the organic solvent-stable α-amylase strains were isolated only from a few organic solvent-tolerant bacterial species, such as *Thalasso bacillus* sp. LY18[27], *Bacillus tequilensis*[28], *Geobacillus thermoleovorans* KIC[29] and *B. subtilis*[30]. The optimization of amylase production is necessary for industries, and the optimal production of enzymes from microorganisms depends on various nutritional and environmental factors, such as carbon and nitrogen sources, pH, temperature, and the incubation period. The requirement of nutritional conditions and physico-chemical properties differ from organism to organism, for their growth and enzyme synthesis[31-34]. Even small improvements in biotechnological enzyme production processes have resulted in great commercial success. Towards this line, the present study addresses the isolation of an organic solvent-stable bacterial strain, and its enhanced production of amylase under sub-merged fermentation.

Materials and Methods

Isolation and screening of organic solvent-stable amylase-producing microorganisms

Organic solvent-stable bacterial strains were isolated from the soil and water samples, according to the previously established methods[18,35]. One gram of soil sample (collected from Incheon, South Korea) was mixedwith10 mL of sterile water by shaking, and 5 mL of the suspensions were added to 250 mL bottles containing 25 mL of LB broth, supplemented with 15% benzene. Culture vessels were sealed with chloroprene rubber stoppers to prevent the evaporation of the organic solvent, and then incubated at 37°C for 3 days on a shaker at 180 rpm. About 5 mL aliquots of these cultures were then transferred into the fresh media and cultured again, under the same conditions. These cultures were diluted and plated onto starch nutrient agar (starch 1 g/L; peptone 5 g/L; yeast extract, 1.5 g/L; beef extract 1.5 g/L; NaCl 3.5 g/L; agar, 20 g/L), without organic solvents. The plates were then incubated at 37°C for 36 h to screen for amylase-producing strains. These strains were purified and screened again on starch nutrient agar plates for further confirmation.

Selection of a highly potent organic solvent-stable strain

Bacteria were inoculated in 25 mL of LB medium and incubated at 30°C for 4 h on a shaker at 180 rpm. Approximately 0.5 mL of this culture was transferred into 50 mL of production medium (starch 10 g/L; peptone 10 g/L; yeast extract 20 g/L; KH_2PO_4 0.1 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; $CaCl_2 \cdot 2H_2O$0.1 g/L; $FeSO_4 \cdot 7H_2O$0.02 g/L; pH 8.0). The inoculated flasks were incubated at 37°C for 48 h on a shaker at 180 rpm. After incubation, the culture was centrifuged for 10 min at 10000 rpm and the temperature was maintained at 4°C.

To obtain a strain highly capable of producing organic solvent-stable amylase, the strains isolated from starch nutrient agar plate were further screened using an organic solvent (5% benzene). The solvent was added to 1 mL of the supernatants, and the tubes were covered with aluminium foil. These mixtures were incubated at 37°C for 24 h on a shaker at 100 rpm. The residual amylase activity was measured. The strain AMY02 was selected based on the starch nutrient agar plate screening, and on solvent stability tests.

Identification of the selected strain by 16S rRNA sequencing

The selected strain was identified by 16S rRNA sequencing. Genomic DNA was extracted using a genomic DNA purification kit (Promega, USA), and then used as a template to amplify 16S rRNA sequences by PCR, using the universal 16S rRNA gene primers: 8-27F, 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1472R: 5′-TACGGYTACCTTGTTACGACTT-3′. The PCR products were then sequenced, and the obtained16S rRNA gene sequences were compared to other nucleotide sequences by BLAST (www.ncbi.nlm.nih.gov/blast). Finally, the phylogenetic tree was constructed using the neighbour-joining method.

Optimization of solvent-stable amylase release under sub-merged fermentation

Amylase production was assessed using different culture media at 24 h intervals, for the incubation time up to 96 h. Cell-free supernatants were collected every 24 h, the following centrifugation for 10 min at 10000 rpm, maintained at 4°C and the supernatants were used for the determination of amylase activity. Different carbon sources (glucose, glycerol, starch, and sucrose) were used at 1% level in the growth media. The carbon sources were sterilized separately and added aseptically to the autoclaved media. The amylase activity was determined at different pH values ranging from 6.0 to

10.0 (adjusted prior to autoclaving), and at temperatures are ranging from 20-50°C.

Amylase assay

The amylase activity was determined by measuring the amount of reducing sugars released during the starch hydrolysis, using 3, 5-dinitrosalicylic acid (DNS) method[36]. A 475 µL of 1% soluble starch in 20 mM Tris-HCl (pH 7.0), containing 0.25 M NaCl and 25 µL of the suitably diluted enzyme solution was incubated at 45°C for 15 min. After incubation, the reaction in the mixture was stopped by adding 500 µL of DNS solution, and the mixture was boiled for 5 min. After cooling the mixture down to 25°C, it was diluted 4 times with distilled water, and the absorbance was measured at 540 nm. One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar equivalent to maltose per minute.

Influence of organic solvents on the stability of crude amylase

Crude amylase from the supernatants was filtered through a 0.22 µM membrane. The enzyme solutions were placed in screw-capped tubes, and mixed with the following organic solvents: benzene, chloroform, methanol, 2-propanol, toluene, and o-xylene[4,15]. All solvents were used at a final concentration of 20%. These mixtures were incubated at 30°C for 2 weeks on a shaker at 100 rpm. After incubation, each sample was carefully withdrawn from the solution or aqueous phase in case of water-immiscible solvents. Residual amylase activity was determined as described above. The controls contained the enzyme solution but lacked the organic solvents. Enzyme stability was expressed as the amylase activity relative to the control.

Results and Discussion

Isolation of organic solvent-tolerant bacteria

Microbes can be isolated from varied extreme conditions and attested to have the unique potentials with the specific substrates[37-44]. In the current study, to obtain the organic solvent-tolerant bacteria the soil and water samples were collected, the organic solvent (benzene) was added to the culture media before the incident microbial growth; this enabled the organic solvent-tolerant bacteria to adapt and grow. After 48 h of incubation at 37°C, the cultures were spread onto the starch nutrient agar plates and incubated under the same conditions. The isolated bacterial strains were further subjected to the screening process for the amylase production under the stress with organic solvent.

Screening and selection of organic solvent-tolerant amylase producing strains

The organic solvent-stable amylase-producing bacterial strains were selected based on the formation of a clearance zone, due to the hydrolysis of starch in the nutrient agar media. Out of 18 isolated strains, only 6 strains were displayed the clearance zones, which were indicative of the presence of amylase activity. The positive strains from this basic screening were transferred again onto the same media for reconfirmation. Of the six strains, the strain AMY02 produced the largest clearance zone, indicating that it was the highest producer of amylase (Table 1 & Fig. 1). In addition, these strains were also evaluated for amylase activity by spectrophotometry measurment, and the solvent tolerant ability. The obtained results were also showed that the strain AMY02 produces amylase with high activity (Table 1), this strain was selected for further studies.

Identification of the organic solvent-tolerant amylase-producing strain

The 16S rRNA gene was amplified from the selected AMY02 strain by PCR, after which the PCR products

Table 1 — Screening for amylase activity and organic solvent stabilities by the isolated strains.

Isolated strain	Plate assay (mm)	Spectrophotometric method	Solvent stability
AMY01	4	++	-
AMY02	5.5	+++	+++
AMY03	4	++	++
AMY04	3	+	+
AMY05	1	+	-
AMY06	5	++	++

"+++" indicate high activity; "++" moderate activity; "+" low activity; "-" no activity

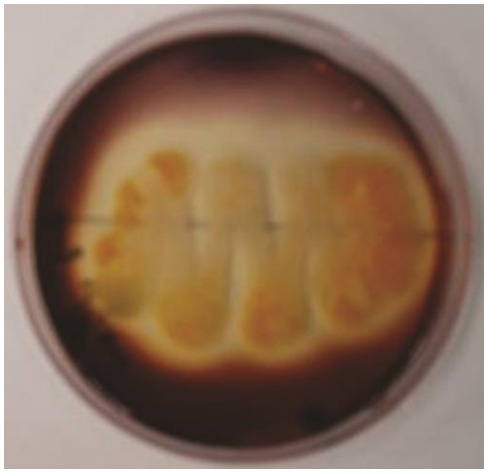

Fig. 1 — Zone of clearance due to the hydrolysis of starch by strain AMY02. Apparent cleared regions are indicated by the arrow

were purified, and used for sequencing. The obtained sequence was compared with other available sequences in the NCBI database. The sequence (1486 bp) of this strain was highly homologous with that of *Bacillus amyloliquefaciens* (Fig. 2). Therefore, the identified strain was designated as *B. amyloliquefaciens* AMY02. The phylogenetic tree was constructed using the neighbour-joining method, and the extracted results revealed that the strain was a part of the cluster within the genus *Bacillus* (Fig. 2).

Optimization of organic solvent-stable amylase release under sub-merged fermentation

Many researchers reported that the media components and physico-chemical properties play an important role in the production of enzymes by bacteria[6,28]. Therefore, we tested the selected strain for amylase production using three different media, under different incubation periods. The maximum amylase production was obtained at 48 h of incubation in the production media; then, the enzyme production was decreased due to the nutrient depletion or the feedback inhibition (Fig. 3). Enzyme activity was seen in nutrient broth and LB broth, at 48 and 72 h of incubation, respectively. However, the enzyme activity in both cases was lower than that in the production media. The other studies also confirmed that the amylase production was the highest after 48 h of incubation[22]. For further studies on amylase production, production media was chosen, and the incubation time was desired to be 48 h. To enhance the production of amylase, different nutritional and physico-chemical properties in the media were further optimized.

Many researchers already have confirmed that physico-chemical properties (pH and temperature) are also involved in the production of enzymes[6,28,32]. In the present study, the highest amylase production was seen at a pH value of 7.0 (Fig. 4A). However, the enzyme activity was greatly reduced when the pH was increased to level between 9.0 and 10.0. Other studies are also supported to claim that the condition at pH 7.0 was favourable for the maximum amylase production[28,45]. The selected strain was cultured on the production media and had a pH of 7.0; this was incubated for 48 h at various temperatures between 20 and 50°C. The strain was able to grow and produced amylase at all the tested temperatures; however, the highest production of

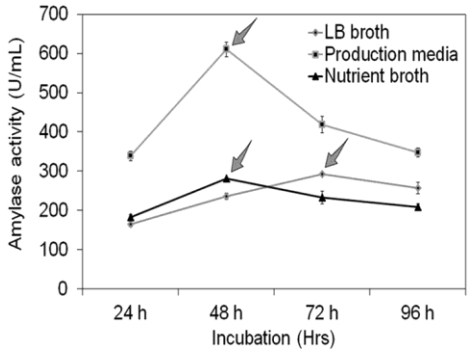

Fig. 3 — Influence of different media on organic solvent-stable amylase production. Each value represents the mean of three experiments, and the error bars indicate ± SD. The highest release is indicated by the arrow

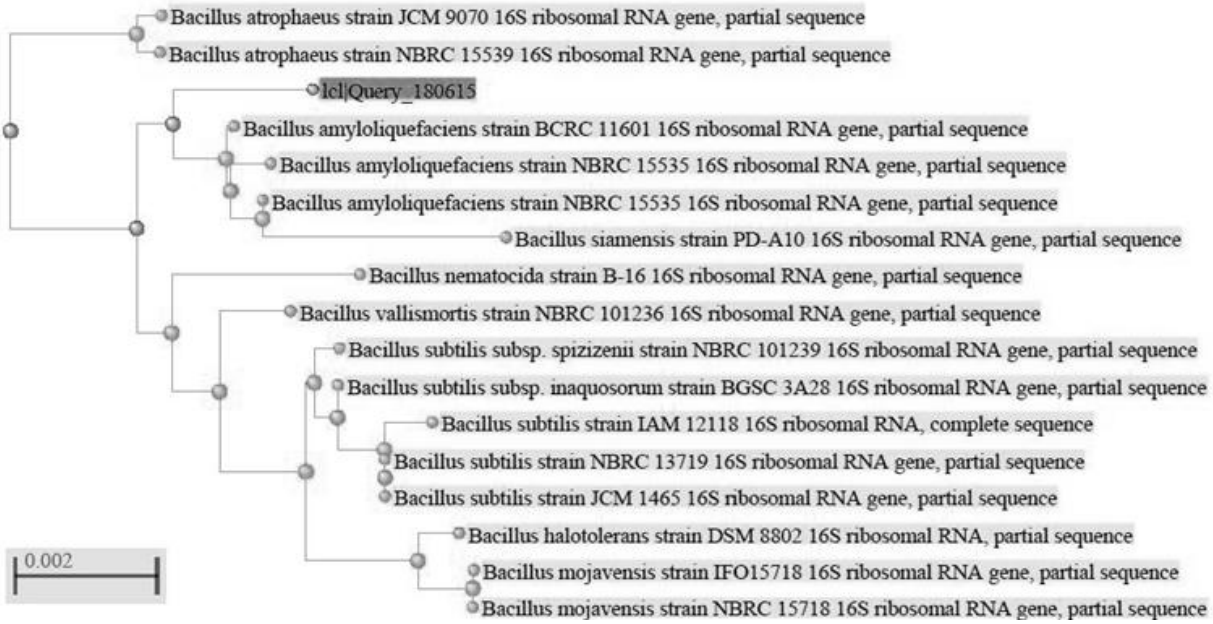

Fig. 2 —Phylogenetic tree showing the relationship between strain AMY02 and other *Bacillus* species

amylase was noticed when the incubation temperature was desired at 30°C (Fig. 4B). Approximately 80% of the enzyme activity was lost at 50°C.

Various carbon sources were tested in the production media to enhance the amylase production. The amylase production was the highest when starch was amended as the carbon source (Fig. 5A). The enzyme production was greatly repressed when the sucrose was used as the sole carbon source. When other two carbon sources, glucose and glycerol were used separately, a moderate level of amylase production was noticed. This result clearly confirmed that starch acts as an inducer for amylase production. Due to the higher rate of amylase production with starch, the amylase production was also assessed in the presence of different concentrations of starch (0.5 to 2%). Amylase production was found to be the highest when 1.5% starch was used (Fig. 5B). The enzyme activity decreased slightly when the concentration of starch was increased to 2%. A similar trend in amylase production by several bacterial species under differing starch concentrations was previously reported[25,28]. Finally, under the optimized media conditions, amylase production was 2.04-fold higher than under the original media composition and conditions were used.

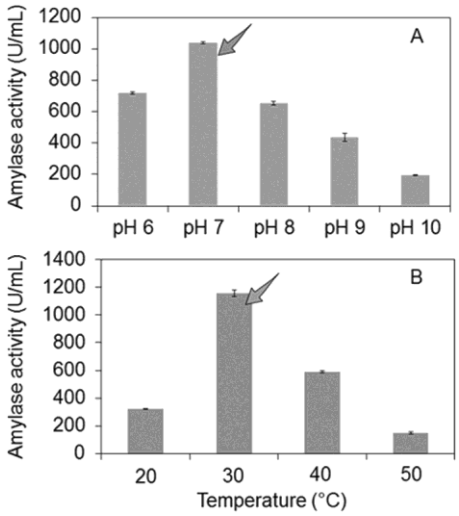

Fig. 4 — (A) Effects of pH; and (B) temperature on organic solvent-stable amylase production. Each value represents the mean of three experiments, and the error bars indicate ± SD. The highest amylase release is indicated by the arrow

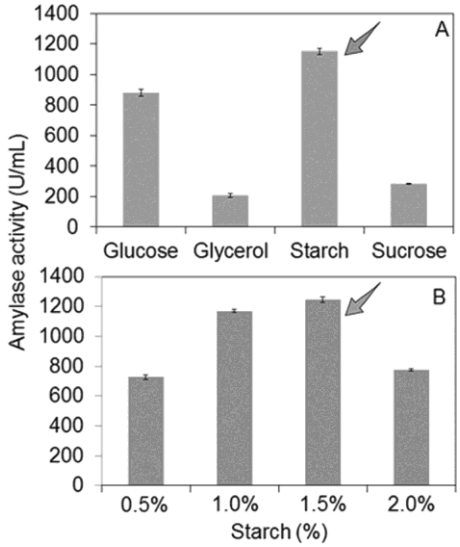

Fig. 5 — (A) Influence of various carbon sources; and (B) starch concentrations on organic solvent-stable amylase production. Each value represents the mean of three experiments, and the error bars indicate ± SD

Stability of crude amylase in various organic solvents

The advantages of biocatalysts in organic media include the increased solubility with hydrophobic substrates, elimination of microbial contamination, and the ability to reuse the catalysts[18,19,29,46]. Although most bacterial enzymes are denatured in the presence of organic solvents, even at 1% concentration, some bacterial strains were able to produce organic solvent-stable enzymes after several adaptations[6,12]. Recently, researchers reported the production of organic solvent-stable proteases and lipases from *Bacillus* species under the high concentrations of organic solvents[6,16,18]. In the present study, the effects of organic solvents on the stability of amylase were evaluated by using various organic solvents at a concentration of 20%, for the

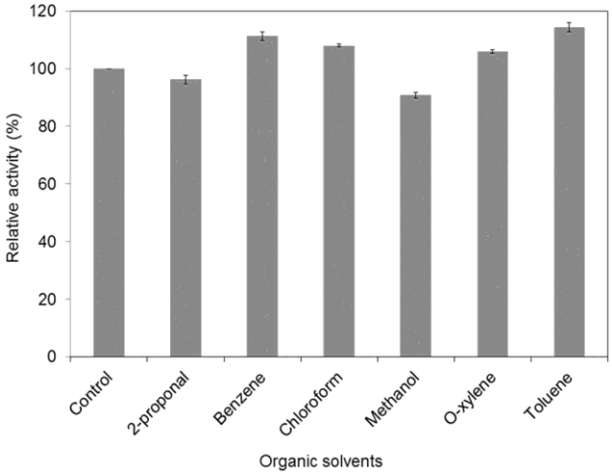

Fig. 6 — Influence of various organic solvents [2-propanol (–0.24), benzene (2.0), chloroform (2.0), methanol (–0.76), O-xylene (3.1), toluene (2.5) on the stability of crude amylase release. Each value represents the mean of three experiments, and the error bars indicate ± SD. The highest amylase production is indicated by the arrow

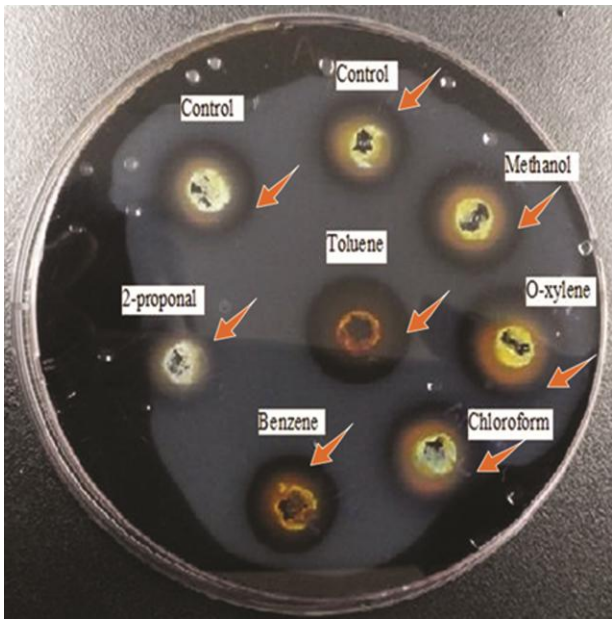

Fig. 7 — Influence of amylase stability in various organic solvents at 20% concentrations based on a plate assay. The apparent cleared regions are indicated by the arrow

continuous period of 2 weeks. The residual enzyme activity was then measured. The enzyme was not only stable in the presence of organic solvents such as benzene, chloroform, O-xylene and toluene, but its activity was also found to increase (from 6 to 14%), compared to that of the control (Fig. 6). However, the activity decreased to 4% and 9%, in the presence of 2-propanol and methanol, respectively. These results clearly confirmed that the enzyme activity was decreased when the log P_{ow} value was less than 2.0, and the enzyme had a greater stability and increased activity while the log P_{ow} value was above 2.0. Organic solvents with low log P_{ow} values (1.5-4.0) are more toxic than those with higher log P_{ow} values[45], however, organic solvents with log P_{ow} values lesser than 2.0 are not considered to be favourable for the biocatalyst action. Therefore, this enzyme will be greatly useful as a biocatalyst in non-aqueous media. Enzyme stability was also confirmed by using the starch nutrient agar plates (with starch as the substrate) (Fig. 7). The result showed clearance zones apparently, indicating the hydrolysis of starch, the level of amylase activity, and its stability in the presence of organic solvents.

Conclusion

The recovery of potential strains tolerates the extreme conditions are welcomed due to their unique applications in different sectors. Herein, a new organic solvent-tolerant bacterial strain was isolated and identified as *B. amyloliquefaciens* AMY02 and this strain can produce an organic solvent-stable amylase in the presence of benzene. Further, the prevailing conditions were optimized to enhance the level of organic solvent-stable enzyme secretion. The yield of amylase has been found to increase 2.04-fold under the optimized media conditions, compared to the amount under the original media condition. The organic solvent-stable amylase was active and stable in the presence of most of the tested solvents even at 20% level. This organic-tolerant amylase from *B. amyloliquefaciens* AMY02 can be utilized as a biocatalyst in non-aqueous media for downstream industrial applications.

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Conflict of interest

All authors declare no conflict of interest.

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