

Original Research Article

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## Development of Cell Disruption Strategy for Enhanced Release of Intracellular Xylose Reductase from *Pseudomonas putida* BSX-46

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Xylose reductase (XR) is responsible for biotransformation of xylose to xylitol and it has gained significant position amongst industrial enzymes due to its role in meeting the demand of xylitol by utilizing agriculture waste. Xylitol is a rare pentose sugar alcohol having a number of therapeutic and pharmaceutical applications. There is a paradigm shift for biotechnological production of xylitol over conventional chemical method. Amongst biotechnological methods, enzymatic method is efficient over whole cell method for industrial xylitol production due to increased product yield and easy recovery of purified product. But, an efficient cell disruption strategy is critically required for the efficient recovery of intracellular XR from the microbial cell. The focus of present study is to test various physical, chemical and enzymatic methods for the disruption of cells of novel isolated *Pseudomonas putida* BSX-46 for maximum release of xylose reductase. Amongst all the methods adopted, sonication treatment given to cells pretreated with EDTA and  $\beta$ -mercaptoethanol was found to be most effective for maximum release of XR with an activity of  $48.70 \pm 0.05$  IU/mg of cells. The findings from the present study can result in development of an efficient method for making use of XR for industrial production of xylitol.

### Introduction

Xylose reductase (XR, EC 1.1.1.21) belongs to aldo-keto-reductase enzyme (AKRs) which catalyzes the reversible conversion of xylose into xylitol by utilizing NADPH or NADH as coenzyme. Xylose reductase is an intermediate enzyme involved during the metabolism of xylose in all the microorganisms including bacteria, yeast, molds and algae (Zheng et al., 2014; Rafiqul and Sakinah, 2015; Zhang et al., 2015). In most of the microorganisms, NADPH is more preferred as co-enzyme with  $K_m$  of  $4.8 \mu\text{M}$  and  $K_{cat}$  of  $21.9 \text{ s}^{-1}$  as compared to NADH with  $K_m$  of  $25.4 \mu\text{M}$  and  $K_{cat}$  of  $18.1 \text{ s}^{-1}$  (Lunzer et al., 1998).

Xylitol is an industrially important alcoholic sugar which shows various promising industrial applications due to its properties like low calorie, negative heat of dissolution, stimulation of flow of saliva without decrease in pH, improvement in taste and color of food and bakery products, metabolism independent to insulin, remineralization of carious lesions, ability to retain moisture and stabilizing nature to prevent protein denaturation (Barathikannan and Agastian, 2016; Lugani et al., 2017). "GRAS" (Generally Recognized as Safe) status has also been given to this alcoholic sugar by Food and Drug Administration, USA (Aguiar et al., 1999).

There is a sudden increase in global market of xylitol due to its wide fruitful applications and it is estimated to be USD1 billion by 2020. Most of the current industrial demand of xylitol is met by chemical method by reduction of D-xylose in the presence of nickel catalyst at elevated temperature and pressure (Granstrom et al., 2007). However, high cost and low yield by chemical method makes this process uneconomical. This results in paradigm shift in adapting biotechnological processes for large scale production of xylitol under ambient conditions. Therefore, the current focus of researchers is towards the biotechnological processes for xylitol production by utilizing whole cell or by extracting the intracellular XR.

A large scale of research has already been conducted by using XR containing various yeast strains for the production of xylitol. However, mere work has been done with microorganism other than yeast for xylitol production. Mass transfer is the major limitation with whole cell use, which decreases the efficiency of enzyme. The other drawbacks associated with the use of whole microbial cells include huge water consumption, cell recycling and downstream processing problems. These limitations can be overcome by using purified XR enzyme, which increases both the enzyme efficiency and reusability for xylitol production due to less energy consumption, easy product recovery, high yield and productivity.

The cell wall of microorganisms provides protection to the cell from osmotic lysis and prevents the release of intracellular content, hence, an efficient cell disruption method is required for the destruction of rigid cell wall mainly peptidoglycan in Gram negative bacteria (Harrison, 1991; Middelberg, 1995). There are various physical, chemical and enzymatic cell disruption methods, but the choice of method is based on the type of

microorganism, composition of its cell wall and intracellular location of enzyme (Saptarshi and Lele, 2010). The first step for downstream processing of an intracellular enzyme is the cell disruption and therefore, a feasible cell disruption method with low operating cost and high product recovery should be developed to make it economically viable at large scale. The aim of present study is to evaluate the effect of various physical, chemical and enzymatic cell disruption methods on *Pseudomonas putida* BSX-46 cells for the release of intracellular XR.

## Materials and Methods

### Culture maintenance

*Pseudomonas putida* BSX-46 cells were maintained on nutrient agar slants containing peptone (5 g/L), NaCl (5 g/L), beef extract (1.50 g/L), yeast extract (1.50 g/L) and agar (20 g/L) with pH adjusted to 7.0.

### Production of xylose reductase

The inoculum was prepared in the Erlenmeyer's flask (250 mL) containing 100 mL of medium comprising peptone (3 g/L) and dextrose (5 g/L) with an initial pH of 7.0 by incubating at 37°C for 12 h under shaking conditions at 150 rpm on a rotatory shaker. XR production was carried out in the Erlenmeyer's flask (250 mL) containing the fermentation medium (50 mL) comprising peptone (3 g/L) and xylose (5 g/L) with pH 7.0 followed by inoculation with 10% (v/v) inoculum and incubating at 37°C for 48 h under shaking conditions (150 rpm).

### Cell disruption of *Pseudomonas putida* BSX-46

After the completion of fermentation process, the bacterial cells were harvested at 500 g for 10 min at 4°C in a refrigerated centrifuge

(5840R, Eppendorf, Germany). The cell biomass was washed twice with phosphate buffer (pH-7.0) to remove any media components and resuspended in the same buffer to make appropriate cell concentration. Different cell disruption techniques including physical, chemical, enzymatic methods and their combinations were tested for disruption of *P. putida* BSX-46 cells for the release of XR.

### **Physical methods**

The following physical methods were tested for disruption of *P. putida* BSX-46 cells for the extraction of intracellular XR enzyme:

#### **Ultra-sonication**

Cell suspension of 10 g/L was subjected to sonication treatment using Ultrasonicator (20 KHz, VCX 500, Sonics, USA) by adjusting the pulse rate to 10 sec on and 10 sec off at different amplitude (50%, 70%) and time intervals (10, 15, 20 min). The distance of sonicator probe and container having cell suspension was maintained at 1 cm. To minimize the protein losses due to excess heat formation, the ultrasonication treatment was carried out in ice bath. After the completion of sonication, the suspension was centrifuged at 5000 g for 10 min at 4°C (5840R, Eppendorf, Germany) and supernatant was analyzed for XR activity.

#### **Pestle-mortar**

The cell slurry was prepared by mixing cell suspension (10 g/L) and sand at different ratios (1:0.25-1:1) followed by grinding of slurry using pestle and mortar for 10 min. The process was carried out in ice bath to prevent loss of enzyme activity due to heat generation. After centrifugation (5000 g for 10 min at 4°C) of grinded cell suspension, supernatant was analyzed for XR activity.

Thereafter, the optimized ratio of cell suspension to sand was grinded for different time intervals (5-20 min) to evaluate the effect of treatment time on cell disruption by estimating the XR activity in the supernatant.

#### **Freeze thawing**

The cell pellet suspended in 100 mM phosphate buffer (pH-7.0) was incubated at -20°C for different time intervals (10-30 min) and after each incubation, the thawing treatment was given to the cells by placing the cell suspension at 30°C for 10 min in water bath. Three cycles of freezing and thawing treatment was given to each vial containing cell suspension. After completion of process, samples were centrifuged (5000 g for 10 min at 4°C) and supernatant was analyzed for detecting XR activity.

#### **Glass beads**

The cell suspension (10 g/L) was mixed with glass beads (0.4 mm) in different ratios (1:0.5-1:4) for 5 min on a vortex mixer to study the effect of concentration of glass beads on bacterial cell disruption. XR activity was estimated in the supernatant to check the effect of glass beads on the extraction of intracellular enzyme.

#### **Silver nanoparticles**

Silver nanoparticles were synthesized by chemical reduction method (Sileikaite et al., 2006). The solutions used in the study were prepared in double distilled deionized water. About 50 mL of silver nitrate ( $1\times 10^{-3}$  M) was heated to boil followed by slow addition of 5 mL of tri-sodium citrate (1%, w/v) through vigorous mixing. The solution was heated until the appearance of pale yellow color, indicating the formation of colloidal silver nanoparticles. The synthesis of silver nanoparticles was confirmed by UV-Vis

Spectrophotometer (Shimadzu, UV-1800) due to their characteristic of optical absorption spectrum in UV-Vis region (Wang et al., 2007). The size of synthesized silver nanoparticles was further confirmed by Zetasizer (Horiba Nanoparticle Analyser, SZ-100).

The active culture suspension of *P. putida* BSX-46 cells (10 g/L) was mixed with synthesized silver nanoparticles solution for 5 min at different concentrations (100 - 5000 µg/mL). The mixture was vortexed at 5000 g for 5 min at 4°C. Thereafter, the treatment time of bacterial cells with optimized concentration of silver nanoparticles was also varied by treating the cells for different time intervals (2-20 min). Then the mixture was centrifuged at 5000 g for 5 min at 4°C, followed by estimation of XR activity in the supernatant.

### **Chemical method**

*P. putida* BSX-46 cells(10 g/L) were incubated with EDTA (Ethylenediaminetetraacetic acid) and β-mercaptoethanol separately with different concentrations (1-5 mM) for 1 h at -20°C. After incubation, the cells were separated through centrifugation at 5000g for 10 min at 4°C and xylose reductase activity was detected in the supernatant. The optimized concentration of EDTA and β-mercaptoethanol was also used in combination to estimate their effect on cell disruption for releasing intracellular enzyme.

### **Enzymatic method**

The cells of *P. putida* belong to Gram negative cells, which possess thin cell wall with outer membrane containing lipopolysaccharide and metal ions. Lysozyme hydrolyzes peptidoglycan layer of cell wall by breaking β 1-4 glycosidic bond between N-

acetyl glucosamine and N-acetyl muramic acid. The cells of Gram negative bacteria become susceptible to lysozyme in the presence of EDTA, which chelates metal ions of outer membrane. Therefore, *P. putida* BSX-46 (10 g/L) cells were mixed with different concentrations (10-35 µL/mL) of lysozyme solution (Sigma) containing EDTA (3mM) and incubated at 30°C for 1 h. The disrupted cells were then separated from lysozyme solution through centrifugation at 5000 g for 10 min at 4°C and XR activity was detected in the supernatant.

### **Combination of physical and chemical methods**

Although sonication is an efficient mechanical method for disruption of bacterial cells, however, to make this method more efficient, its combination with chemical pretreatment of cells was tested for maximum release of intracellular XR. In the current study, pretreatment was given to the cells with EDTA (2mM) solution prepared in phosphate buffer (pH 7.0) for 1 h. Thereafter, cells were subjected to another chemical treatment by suspending them in phosphate buffer (pH-7.0) containing β-mercaptoethanol (3 mM) and incubated for 1 h at -20°C to further increase the permeability of cells. After these chemical treatments, the permeabilized cells were further disrupted with optimized sonication treatment (70% amplitude, pulse rate of 10 sec on and 10 sec off for 15 min). After centrifugation (5000 g for 10 min at 4°C) of treated samples, the supernatant was analyzed for detecting XR activity.

### **Combination of physical and enzymatic method**

The enzymatic method has shown efficient results for maximum release of XR than other physical methods tested, hence, its

combination with sonication treatment was also tested. Cells of *P. putida* BSX-46 (10 g/L) were treated with lysozyme solution (20 µL/mL) followed by optimized sonication treatment (70% amplitude, pulse rate of 10 sec on and 10 sec off for 15 min) to estimate the effect of combination of physical and enzymatic methods on cell disruption. Thereafter, the cells were separated as described previously and XR activity was analyzed in the supernatant.

### **Enzyme assay**

XR activity was estimated spectrophotometrically at 340 nm with specific co-enzyme NADPH by the method of Yokoyama et al. (1995). One unit of enzyme activity is defined as the amount of enzyme which results in decrease in NADPH concentration by one µM per min under standard assay conditions.

### **Statistical analysis**

The experimental data obtained after mean from each triplicate set was statistically evaluated using ANOVA from SPSS 16.0 program at a significance level of  $p < 0.05$ .

## **Results and Discussion**

Different cell disruption methods including physical, chemical, enzymatic and combination of two methods with varying parameters were optimized to enhance the extraction of XR from cells of *P. putida* BSX-46. XR activity on optimized parameters using different methods are summarized in Table 1.

### **Cell disruption through physical methods**

Various physical methods (ultrasonication, pestle mortar, freeze thawing, glass beads and silver nanoparticles) were used for the extraction of intracellular XR from isolated *P. putida* BSX-46. The impact of these methods in terms of XR activity is discussed.

### **Ultrasonication**

The effect of ultrasonication on disruption of *P. putida* BSX-46 cells for the release of XR was studied by varying the sonication parameters including pulse rate, sonication time and amplitude. The relative XR activity at different parameters is shown in Figure 1. It was found from the results that maximum release of XR with activity of  $34.49 \pm 0.31$  IU/mg of cells was observed when the sonication was done with pulse rate of 10 sec on and 10 sec off at 70% amplitude for 15 min. However, XR activity was decreased when the sonication time was increased to 20 min. The decrease in activity after optimum time may be due to fragmentation of desired protein with increasing the sonication time. The low XR activity obtained at low amplitude below optimum value may be due to inadequate effect, which is required for breaking the cells.

### **Pestle-mortar**

The cell suspension was mixed with different ratios of sand to optimize the cell suspension to sand ratio for the maximum release of XR. From the results (Figure 2), it has been concluded that cell suspension to sand ratio of 1:0.5 gave maximum relative XR activity of  $80.54 \pm 0.14\%$  after 10 min compared to control and this treatment of cell disruption was found to be less efficient as compared to ultrasonication method. Further, the effect of treatment time was also tested by using optimized cell suspension to sand ratio and maximum release of XR was achieved when the treatment time was increased to 15 min with relative XR activity of  $87.21 \pm 0.22\%$  (Figure 3). Further increase in time beyond 15 min could not increase the enzyme activity in the extract, which may be due to protein

denaturation occurred due to prolonged treatment time.

### Freeze thawing

Freeze thawing is an efficient method of cell disruption which results in the release of cell content through formation and subsequent melting of ice crystals. The maximum release of xylose reductase was observed at freeze thaw ratio of 2:1 (20 min freezing, 10 min thawing) with relative activity of  $80.54\pm0.43\%$  compared to control (Figure 4). However, no significant difference in xylose reductase was observed by increasing the freezing time, which may be due to extensive damage of cells by forming larger crystals.

### Glass beads

The different ratios of cell suspension to bead volume was used in the current study to evaluate its impact on extraction of XR from *P. putida* BSX-46 cells and maximum XR relative activity of  $94.41\pm1.03\%$  was observed at cell suspension to bead volume of 1:3 (Figure 5). When this ratio was increased to 1:4, the enzyme activity was decreased

which may be due to heat generation during the process resulting in protein denaturation (Middelberg, 1995).

### Silver nanoparticles

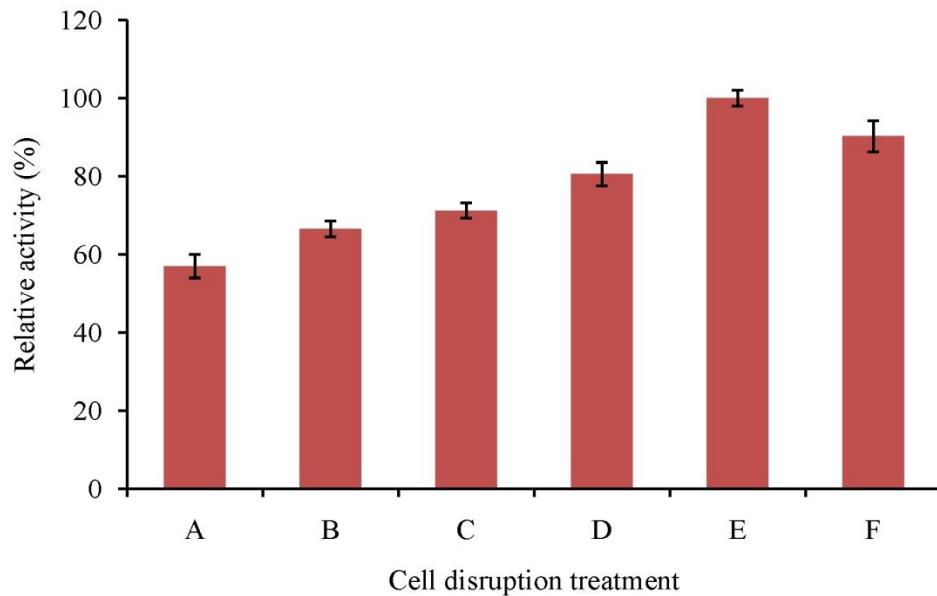
Silver nanoparticles were synthesized by chemical reduction method described previously and they showed pale yellow color with a characteristic plasmon resonance band which was measured by UV-Vis spectroscopy. The absorbance peak of silver nanoparticles was observed at 430 nm, which proved the formation of silver nanoparticles and sharp particle size distribution was found due to symmetrical shape of band. The size of silver nanoparticles was computed through Zeta sizer, which was found to be  $7.8\pm0.3$  nm.

The synthesized silver nanoparticles were further used for bacterial cell disruption by varying its concentration and treatment time. The maximum release of xylose reductase was observed at a concentration of 1000  $\mu\text{g/mL}$  (Figure 6) with relative activity of  $95.71\pm0.02\%$ . However, the concentration of silver nanoparticles above 1000  $\mu\text{g/mL}$  showed decrease in enzyme activity.

**Table.1** Summary of different cell disruption methods with optimized parameters for the extraction of intracellular xylose reductase from *Pseudomonas putida* BSX-46 cells

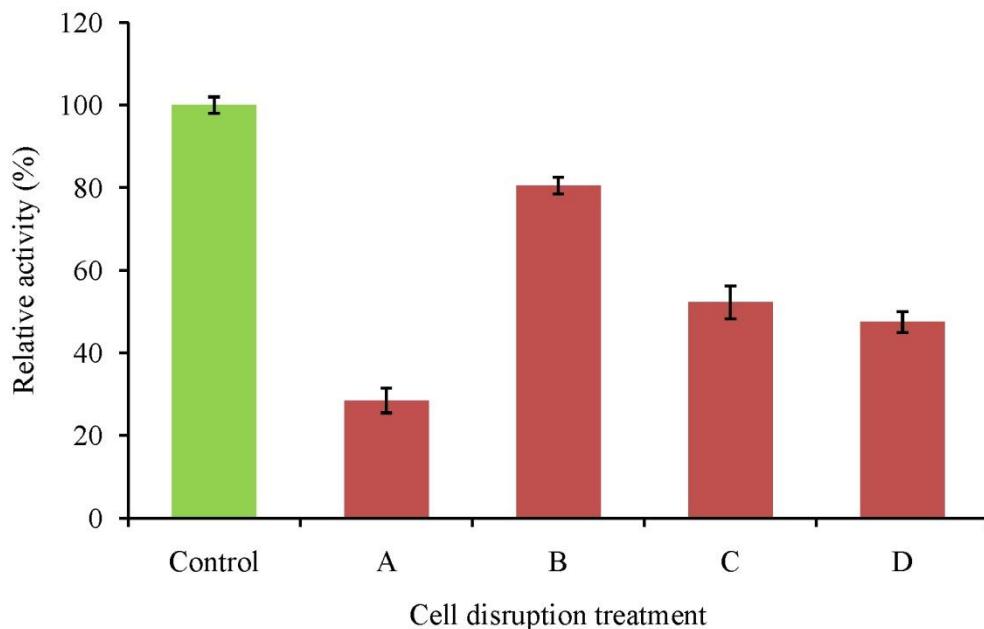
Method	Optimized parameters	Relative XR activity (%)
Ultrasonication	10 sec on, 10 sec off for 15 min at 70% amplitude	$100\pm0.32$
Pestle-mortar	1:0.5 (Cell suspension: sand), 10 min	$80.54\pm0.14$
	1:0.5, 15 min	$87.21\pm0.22$
Freeze-thawing	2:1 (20 min freezing, 10 min thawing)	$80.54\pm0.43$
Glass beads	1:3 (Cell suspension: bead volume), 5 min	$94.41\pm1.03$
Silver nanoparticles	1000 $\mu\text{g/mL}$ , 5 min	$95.71\pm0.02$
	1000 $\mu\text{g/mL}$ , 10 min	$98.94\pm0.32$
Chemical treatment	2 mM EDTA	$115.14\pm1.17$
	3 mM $\beta$ -mercaptoethanol	$120.14\pm0.22$
	2 mM EDTA+ 3 mM $\beta$ -mercaptoethanol	$130.21\pm0.34$
Enzymatic treatment (Lysozyme)	20 $\mu\text{L/mL}$	$118.73\pm0.53$
Ultrasonication+ chemical treatment	10 sec on, 10 sec off for 15 min at 70% amplitude+ 2 mM EDTA+ 3 mM $\beta$ -mercaptoethanol	$141.20\pm1.00$
Ultrasonication+ enzymatic treatment	10 sec on, 10 sec off for 15 min at 70% amplitude+ 20 $\mu\text{L/mL}$ Lysozyme	$128.22\pm0.32$

**Fig.1** Effect of ultrasonication on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



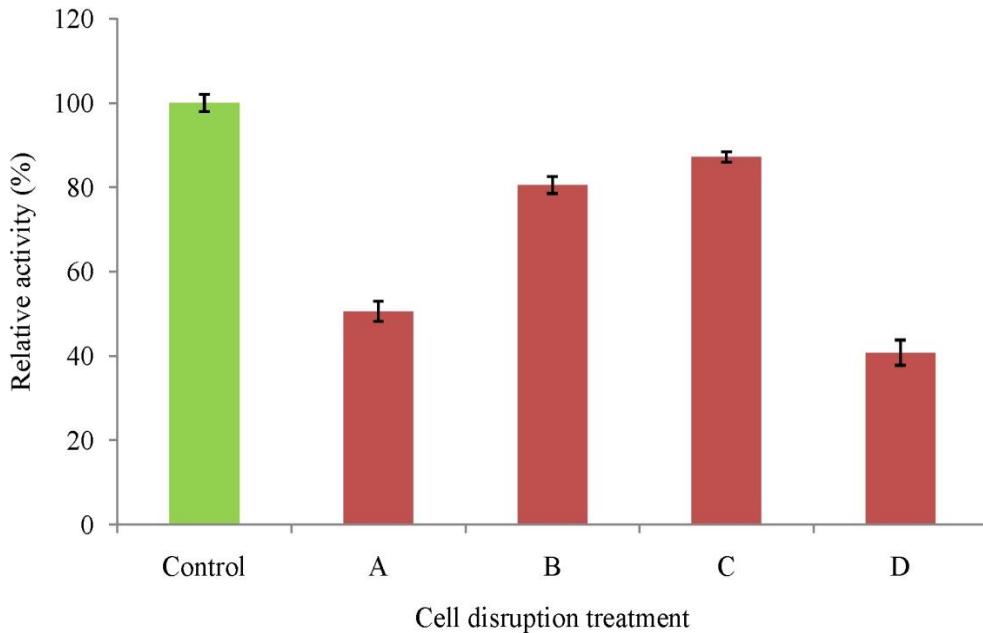
Where, A. Ultra-sonication for 10 minutes, 50% amplitude; B. 15 minutes, 50% amplitude; C. 20 minutes, 50% amplitude; D. 10 minutes, 70% amplitude; E. 15 minutes, 70% amplitude; F. 20 minutes, 70% amplitude, with pulse rate of 10 sec on, 10 sec off in all tests.

**Fig.2** Effect of pestle mortar on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



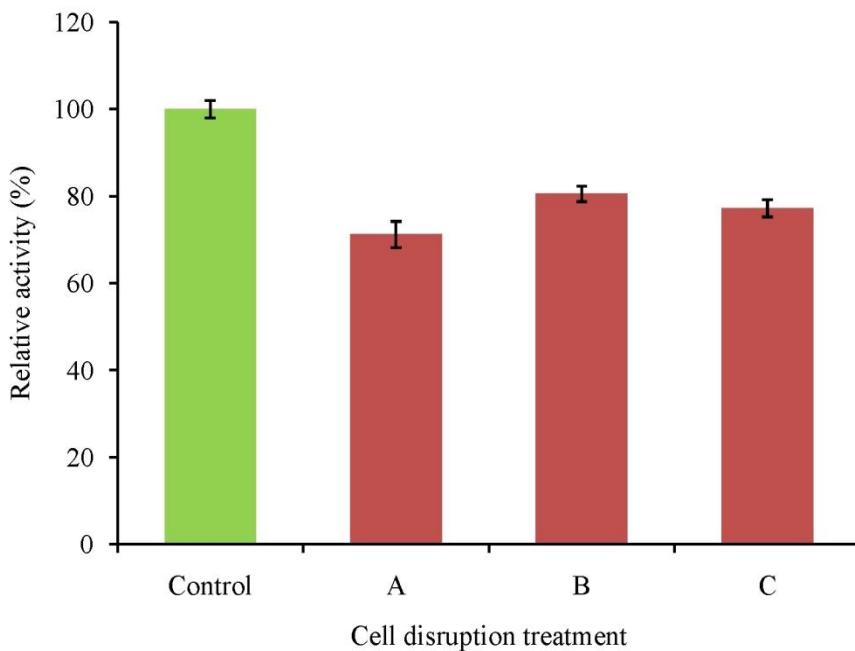
Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; Cell weight: Sand ratio- A.1.0:0.25;B.1.0:0.50;C.1.0:0.75; D.1.0:1.0.

**Fig.3** Effect of pestle mortar treatment time on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



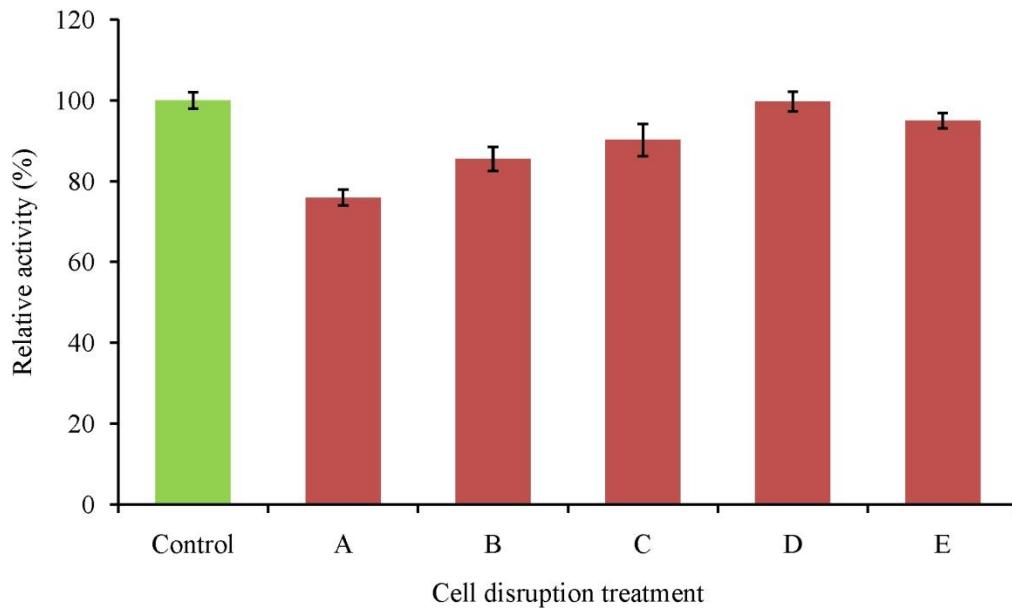
Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; Cell weight: Sand ratio- A. 5 min; B.10 min; C.15 min; D.20 min.

**Fig.4** Effect of freeze thawing on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



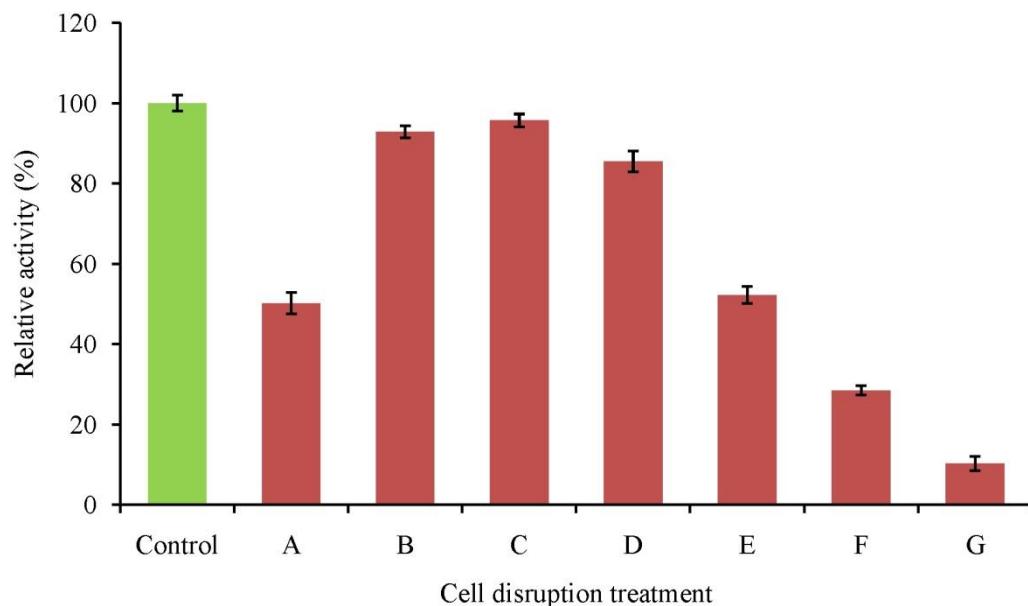
Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; A. 10 minutes freezing, 10 minutes thawing (1:1); B. 20 minutes freezing, 10 minutes thawing (2:1); C. 30 minutes freezing, 10 minutes thawing (3:1).

**Fig.5** Effect of glass beads on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



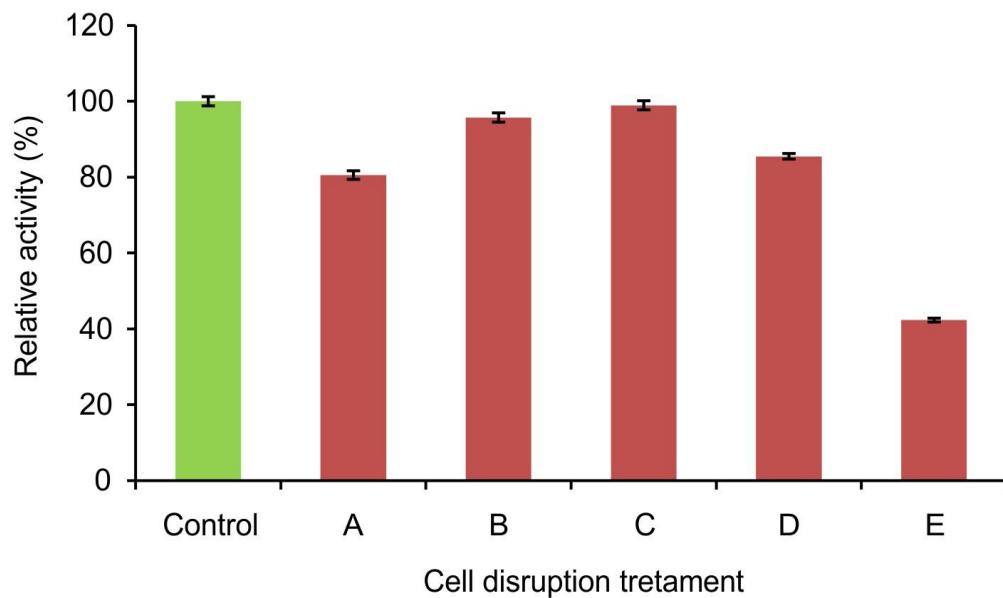
Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; Cell weight: Glass beads ratio- A.1.0:0.5; B.1.0:1.0; C.1.0:2.0;D.1.0:3.0; E.1.0:4.0.

**Fig.6** Effect of silver nanoparticles on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



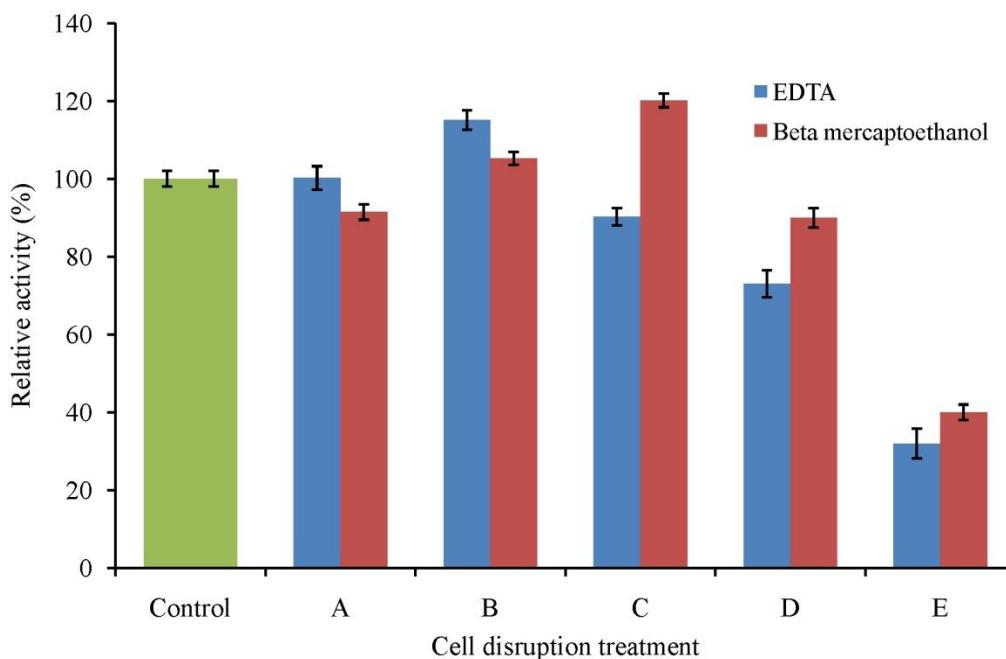
Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; A.100 µg/mL; B. 500 µg/mL; C. 1000 µg/mL; D. 2000 µg/mL; E. 3000 µg/mL; F: 3000 µg/mL; G: 3000 µg/mL.

**Fig.7** Effect of treatment time of silver nanoparticles on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



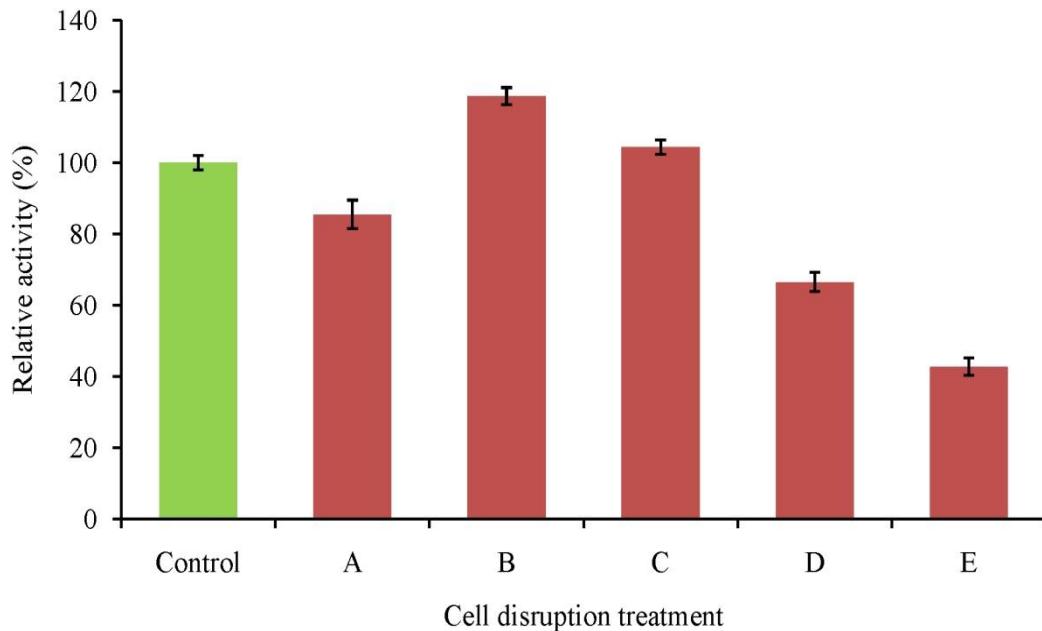
Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; A.2 min; B. 5 min; C. 10 min; D. 15 min; E. 20 min.

**Fig.8** Effect of EDTA and  $\beta$ -mercaptoethanol on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



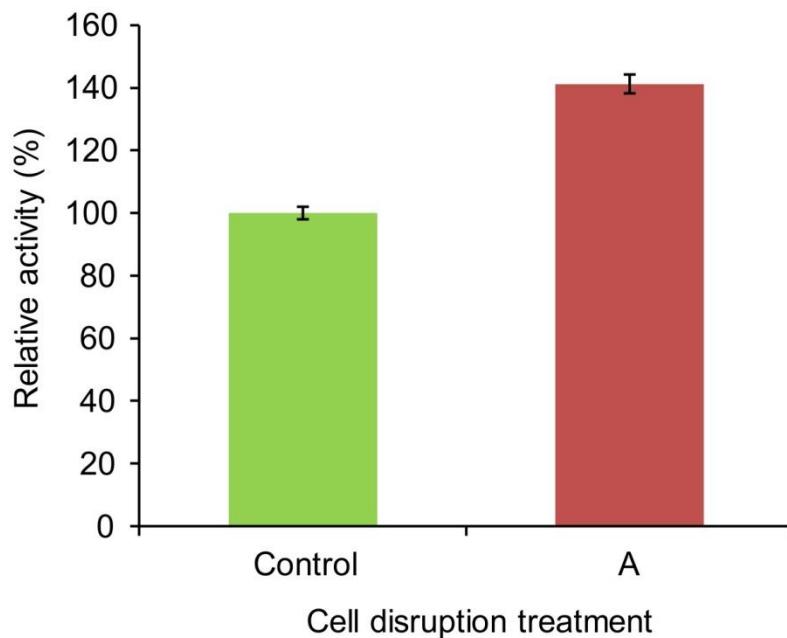
Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; A.1.0 mM; B.2.0 mM; C.3.0 mM; D.4.0 mM; E.5.0 mM.

**Fig.9** Effect of Lysozyme on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



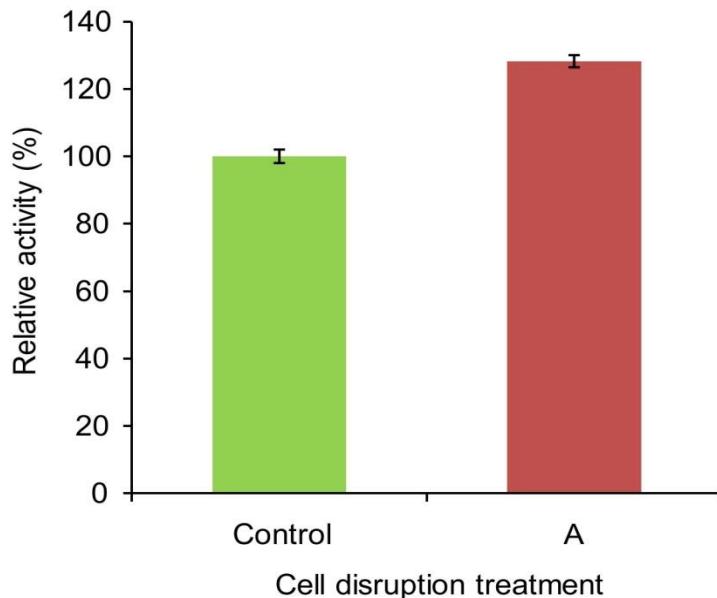
Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; A.10  $\mu$ L/mL; B.20  $\mu$ L/mL; C.25  $\mu$ L/mL; D.30  $\mu$ L/mL; E.35  $\mu$ L/mL.

**Fig.10** Effect of combination of physical and chemical method on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; A. Pretreatment with 2mM EDTA and 3mM beta-mercaptoethanol followed by Sonication (10 sec on, 10 sec off, 15 minutes, 70% amplitude).

**Fig.11** Effect of combination of physical and enzymatic method on release of xylose reductase from *Pseudomonas putida* BSX-46 cells



Where, Control- Ultra-sonication for 10 sec on, 10 sec off, 15 minutes, 70% amplitude; A. Lysozyme treatment (20  $\mu$ l) followed by Sonication (10 sec on, 10 sec off, 15 minutes, 70% amplitude).

Further, the treatment time of silver nanoparticles was optimized and maximum relative XR activity of  $98.94\pm0.32\%$  was recorded after 10 min (Figure 7). Among all the physical methods of cell disruption, the use of silver nanoparticles has shown the comparable results as compared to sonication method for disruption of *P. putida* BSX-46 cells for releasing maximum XR.

#### Cell disruption through chemical treatment

In the current study, *P. putida* BSX-46 cells were treated with chemicals EDTA and  $\beta$ -mercaptoethanol with different concentrations (1-5mM) to estimate their effect on release of intracellular xylose reductase and the maximum relative xylose reductase activity was found with EDTA at a concentration of 2 mM ( $115.14\pm1.17\%$ ) and  $\beta$ -mercaptoethanol at a concentration of 3 mM ( $120.14\pm0.22\%$ ) as compared to control (Figure 8). Further, the initial treatment of cells with 2 mM EDTA followed by treatment with 3 mM  $\beta$ -

mercaptoethanol further enhanced the efficiency of cell disruption with relative XR activity of  $130.21\pm0.34\%$ . It has been evidenced previously that EDTA acts as a chelator and it permeabilizes the membrane of Gram negative bacteria through sequestering divalent cations of outer membrane by loosening their electrostatic interaction with proteins and liposaccharides. Similarly,  $\beta$ -mercaptoethanol enhances the permeabilization of cell wall of Gram negative bacteria. The chemical treatment was more efficient as compared to physical methods due to their synergistic effect for providing both cell disruption and stability effect for retaining tertiary structure of enzyme.

#### Cell disruption through enzymatic method

Lysozyme is very effective for hydrolyzing the membrane of Gram negative bacteria when it is used in combination with EDTA. Hence, this enzyme was used to disrupt cell membrane of *P. putida* BSX-46 cells for the

extraction of intracellular XR and the maximum relative XR activity of  $118.73\pm0.53\%$  (Figure 9) was observed with use of 20  $\mu$ L/mL of lysozyme solution. Thus, enzymatic method is an effective method for the extraction of intracellular proteins from Gram negative cells. Enzymatic method was found to be more efficient as compared to all physical methods but less efficient as compared to chemical method for the disruption of Gram negative cells.

### **Cell disruption through combination of physical and chemical method**

A combination of physical and chemical method was used to check their synergistic effect on cell disruption compared to individual ones. It has been observed that initial permeabilization of bacterial cells with EDTA (2mM) and  $\beta$ -mercaptoethanol (3mM) followed by sonication treatment (70% amplitude, pulse rate of 10 sec on and 10 sec off for 15 min) improves the effectiveness of treatment for disruption of Gram negative cells for maximum release of XR. The XR activity of  $48.70\pm0.05$  IU/mg of cells and relative activity of  $141.20\pm1.00\%$  (Figure 10) was observed by disruption the cells through combination of physical and chemical method and thus this treatment was more effective compared to individual physical treatment and chemical treatment. The increased xylose reductase activity by using two methods may be due to the loosening of cell wall by EDTA and  $\beta$ -mercaptoethanol along with helping in maintaining stability of tertiary structure of enzyme before sonication treatment.

### **Cell disruption through combination of physical and enzymatic method**

The effect of disruption of *P.putida*BSX-46 cells through a combination of physical and enzymatic method was also evaluated. It was found that treatment of cell suspension with

lysozyme solution (20  $\mu$ L/mL) followed by optimized sonication treatment (70% amplitude, pulse rate of 10 sec on and 10 sec off for 15 min) showed the relative XR activity of  $128.22\pm0.32\%$  as compared to sonication method (Figure 11), which was higher compared to individual physical and enzymatic methods. This may be due to the fact that treatment of Gram negative bacterial cells with lysozyme yield osmotically fragile cells without complete destruction of rigid cell wall and further treatment with sonication method results in effective rupturing of bacterial cells.

An efficient cell disruption method is required for the easy extraction of intracellular content and the current study is focused towards the use of different physical, chemical and enzymatic methods and their combinations to develop an efficient strategy for recovery of XR from *P. putida* BSX-46. Previously, various authors have utilized different methods for the extraction of many industrially important enzymes. Sonication method was used for the release of intracellular aspartase from *E. coli* K-12 cells (Singh, 2013). The ultrasonication method was also used effectively by Tangtua (2014) for the extraction of intracellular pyruvate decarboxylase from *Candida tropicalis* TISTR 5350. In a recent study, the cells of *Chlorella vulgaris* were efficiently disrupted by ultrasonication treatment (Huang et al., 2016). Pestle mortar treatment was also used for the release of  $\beta$ -galactosidase from *Kluyveromyces lactis* NRRL Y-8279 (Dagbagli and Goksungur, 2008). Freeze thawing was also employed previously for the release of aspartate carbamoyltransferase from *Helicobacter pylori* by Burns et al. (1997). Similarly, the cells of *Candida mogii* were disrupted by freeze-thawing for the recovery of intracellular xylose reductase (Mayerhoff et al., 2008). In a previous study, cell suspension volume to bead volume of 1:0.5

was found to be the best for release of intracellular lipoic acid from *Saccharomyces cerevisiae* (Jayakar and Singhal, 2012). Silver nanoparticles enhances the permeability of cell membrane of Gram negative bacteria for reducing sugars and proteins (Li et al., 2010). The high efficiency of silver nanoparticles for the release of intracellular pyruvate decarboxylase from *Candida tropicalis* TISTR 5350 has also been reported by Tangtua (2014). The effect of stability of tertiary structure of reductases using EDTA and  $\beta$ -mercaptoethanol has also been reported in a previous study conducted by Sayed et al. (2016). Different workers have utilized various physical, chemical and enzymatic methods for disruption of different microbial cells, however the effect of combination of these methods on cell disruption tested in present study is not reported till date.

It has been concluded from the present study that among all the methods of cell disruption, initial chemical permeabilization of bacterial cells followed by ultrasonication proved to be best and efficient method for the extraction of intracellular XR enzyme. A combination of two or more cell disruption techniques is found to be the method of choice due to less damage of intracellular enzymes. XR is found to be an emerging industrially important enzyme due to its ability for xylitol production.

Most of the previous research on biotechnological xylitol production has been done with microbial whole cells and very little information has been available on use of enzyme based xylitol production. Therefore, the present study on optimizing cell disruption method with novel isolated *P. putida* BSX-46 strain for the recovery of intracellular XR would have industrial importance in future for development of enzyme based process for production of xylitol.

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## Conflict of Interest

The authors declare that they have no conflict of interest in the present publication.

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