Biotechnology Reports 5 (2015) 89–97

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Investigations on ideal mode of cell disruption in extremely halophilic *Actinopolyspora halophila* (MTCC 263) for efficient release of glycine betaine and trehalose

Jayaranjan R. Kar, Rekha S. Singhal *

Department of Food Engineering and Technology, Institute of Chemical Technology, N.P Marg, Matunga, Mumbai 400019, Maharashtra, India

ARTICLE INFO

Article history:
Received 1 October 2014
Received in revised form 11 November 2014
Accepted 15 December 2014
Available online 18 December 2014

Keywords:
Osmotic shock
Morse equation
Actinopolyspora halophila

ABSTRACT

Actinopolyspora halophila produces glycine betaine and trehalose intracellularly in considerable quantities. These biomolecules are commercially important as they have applications in food, pharmaceuticals, and agricultural sector. Development of an efficient cell disruption technique is an important step for the release of these biomolecules. In this study, various cell disruption methods such as chemical, enzymatic, physico-mechanical and physical methods were evaluated. Cell disruption by osmotic shock was found to be the best suited method for *A. halophila* which also has a potential to be industrially scaled up. Cell bursting pressure that is generated during osmotic shock in *A. halophila* was computed using Morse equation and was found to be $\pi = 238.37 \pm 29.54$ atm or 2.35 ± 0.29 kPa. In addition, it was found that osmotic shock followed a first order release rate kinetics in *A. halophila*. The findings can be used for commercially important biomolecules from other halophilic and/or halotolerant microbes.

1. Introduction

Glycine betaine and trehalose are found in many organisms, mainly as osmoprotectants, which protect them in an environment of high salt concentration [1]. Glycine betaine is a quaternary ammonium compound which is amphoteric in nature and is metabolically synthesized from choline or in some cases, by glycine [2]. Trehalose is a disaccharide that is metabolically synthesized from UDP glucose and glucose-6-phosphate [3]. Glycine betaine and trehalose are economically significant molecules as they have a multitude of applications in a variety of fields [4–9]. Natural glycine betaine is industrially isolated from sugar beet molasses and *Ascophyllum nodosum* through various bioseparation techniques [10,11]. Although glycine betaine is chemically produced, the use of natural glycine betaine like Betafin® is advocated over synthetic glycine betaine due to its natural origin and absence of chemical contaminants [12]. Commercial production of trehalose is carried out through fermentation [13], but the potential for newer organisms need to be continuously explored. Hence, their separation and isolation are of commercial importance.

Actinopolyspora halophila produces glycine betaine and trehalose intracellularly [1]. Previous work from our laboratories has shown significantly high production of glycine betaine (9.07 ± 0.25 g/L) and trehalose (2.49 ± 0.14 g/L) by *A. halophila* in acid whey as a growth medium [14]. The cell wall of *A. halophila* is very unique and different from the cell walls of other actinomycetes [15]. *A. halophila* requires at least 12% (w/v) NaCl in liquid medium for its survival [16]. Yamaguchi has classified actinomycetes into five different types depending upon their cell wall composition [17]. However, actinomycetes are generally not classified on the basis of their resistance to salinity [18,19]. As compared to other actinomycetes which have higher concentration of amino acids other than that required for peptidoglycan synthesis in their cell wall composition [20],*A. halophila* cell wall constitutes 70% of peptidoglycan of the total cell wall weight [15]. *A. halophila* cell wall comprises of glutamic acid, alanine, and diaminopimelic acid in a 1:2:1 molar ratio, lipids, D-galactose and D-arabinose [15,16]. Due to its unique cell wall composition, an effective method of cell disruption for substantial release of glycine betaine and trehalose is of utmost importance.

Cell disruption methods have been extensively studied in yeasts, *Escherichia coli* and *Bacillus subtilis* that have industrial importance due to the large volumes of intracellular substances recovered from them, both from the native as well as in genetically modified forms [21]. Very few cell disruption studies have

* Corresponding author. Tel.: +91 22 33612501; fax: +91 22 33611020.
E-mail address: rs.singhal@ictmumbai.edu.in (R.S. Singhal).

http://dx.doi.org/10.1016/j.btre.2014.12.005

beenconducted on microorganisms other than the above mentioned microbes [21]. There are many methods of cell disruption on a lab scale [22], but industrially only few mechanical methods of cell disruption like bead mill, high pressure homogenizer and Hughes press are used extensively due to ease in scale up of the operations and cost effectiveness [23]. Another reason of preferring mechanical methods like bead mill over chemical and enzymatic methods for cell disruption at industrial scale is to avoid the increase in unit operation steps during downstream processing. The enzymes or chemicals used to achieve cell disruption have to be removed from the cell lysate during the purification of the product of interest, thus increasing the cost of production. Physical methods of cell disruption like osmotic shock, freeze–thaw, liquid nitrogen freezing with grinding and nitrogen bomb have the potential to be used on an industrial scale but have limited applicability [21,22,24,25]. In many studies, the extent of cell disruption and the release of intracellular materials have been measured by using indirect methods like protein estimation, carbohydrate estimation, conductance and colorimetric measurements [23,26–28] instead of measuring the actual product of interest. The best way to estimate the extent of cell disruption is by directly estimating the product of interest for which the cell disruption is being performed [28].

An efficient method of 70% ethanol lysis [29], which has been successfully utilized in *E. coli* for release of intracellular glycine betaine was also found to be a good method in *A. halophila.* Cell lysis using 70% ethanol was therefore, used as a benchmark to compare other cell disruption methods. The aim was to identify a cell disruption method for *A. halophila* that can be industrially scaled up and should be energy efficient (in turn cost-effective). These criteria were found in an osmotic shock process. Besides, the kinetics of cell disruption by osmotic shock was studied which has not been previously documented. The approximate intracellular cell bursting pressure that is generated during the osmotic shock was also calculated by measuring the amount of NaCl in the cell lysate (which is the major salt in case of *A. halophila*).

2. Material and methods

2.1. Materials

Materials used in the formulation of media were bought from HiMedia Ltd. (NaCl, NH_4Cl, K_2HPO_4, $CaCl_2{\cdot}2H_2O$, $MnSO_4{\cdot}H_2O$ and glycine) Mumbai, India; Merck ($MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$), India; Sigma–Aldrich (corn steep liquor and $SeCl_4$), India. Solvents and other chemicals were bought from S.D. Fine Chemicals Limited, Mumbai, India. Enzymes used were papain (RM058-HiMedia, India, having an activity of 31,734 TU/g of protein), trypsin (204013-Sisco Research Laboratories, India, having an activity of 2500×10^3 NFU/g of protein), protease (Protex 6L-Genecor, Denmark, having an activity of 580,000 DU/g protein), pancreatin (P-1750, Sigma–Aldrich, India, having an activity of 106,261 U/g protein), and lipase (L3126, Sigma–Aldrich, India, having an activity of 340,745 U/g of protein).

2.2. Biomass production of A. halophila and measurement of dry cell weight (DCW)

Acid whey was provided by small scale *paneer* (cottagecheese) industry in Mumbai, India. Media composition: 21.9 (% w/v)NaCl, 3.38 (% w/v) NH_4Cl, 0.1 M $MgSO_4{\cdot}7H_2O$, 0.1 M K_2HPO_4, 10 mM $FeSO_4{\cdot}7H_2O$, 5 mM $CaCl_2{\cdot}2H_2O$, 5 mM $MnSO_4{\cdot}H_2O$, 6.21 mM $SeCl_4$, 1.5 (% w/v) glycine, 3 (% w/v) corn steep liquor, 94.07 (% v/v) whey and pH of media was adjusted to 8.0. Media with whey was processed [30] with few modifications. Whey media was kept at 100 °C for 20 min. It was centrifuged at $3857 \times g$ for 30 min at 4 °C and then passed through sterile filter of pore size 0.2 μm to filter sterilize the media under sterile conditions. 50 mL of sterile media were transferred into sterile 250 mL Erlenmeyer flasks under sterile conditions. These flasks were incubated at 37 °C, 180 rpm for 96 h. Inoculum was prepared in the same media as mentioned above, and 2% of 72 h old inoculum with 10^5 cells/mL was inoculated into these flasks. The biomass was separated after 96 h from the broth by centrifuging at $3857 \times g$ for 20 min at 4 °C. Cells were then washed twice with a 21.9 (% w/v) salt solution (same concentration as used in media) to get rid of cellular debris. These cells of *A. halophila* were then used for disruption studies. 2 mL of broth was taken in 2 mL Eppendroff tubes (weighed) and centrifuged at $9615 \times g$. The cell pellet was vortexed and washed with 21.9 (%w/v) sodium chloride solutions. This was repeated twice, and the difference in weight was measured for dry cell weight (DCW) after drying the tubes in 60 °C hot air oven for 48 h, where a constant and persistent weight was obtained.

2.3. Analysis of glycine betaine, trehalose and sodium

After disrupting the cells by various methods, the cell lysates were centrifuged and dried. Known amounts of this dried cell lysate were dissolved in distilled water (d/w) or methanol (depending upon the analysis that has to be performed). This solution was then quantified for glycine betaine by a modified UV spectrometric method [31], where samples were subjected to periodide reaction by using 0.5 mL of concentrated sulfuric acid instead of 10 drops and then centrifuged in a swinging bucket centrifuge at $2450 \times g$ for 20 min at 0 °C. Graduated and tipped centrifuge tubes of Borosil glass (15 mL) were used to carry out the periodide reaction. A standard curve developed over a concentration range of 0–50 μg glycine betaine gave a regression equation $y = 0.0119\times$ ($R^2 = 0.9957$), where y was the optical density at 365 nm and x was the concentration of GB in microgram. Trehalose was quantified by a well-established HPTLC method [32]. Sodium analysis was performed in order to calculate NaCl concentration and in turn the total cell bursting pressure by Morse equation for the optimized osmotic shock method. Sodium content of the cell lysate solution was analyzed by Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES) (ARCOS from M/s. Spectro, Germany) using standard curve of sodium ($y = 52058\times$, $R^2 = 0.9996$, where y was the intensity (cps) at 589.59 nm, and x was the concentration of Na in ppm). Cell lysate solutions were diluted appropriately for ICP-AES analysis and prepared in deionized d/w.

$$(\text{Morse equation})\pi = MR^IT \quad (1)$$

where π is cell rupturing pressure generated due to osmotic shock (units in atm or kPa), M is molar concentration of NaCl, R^I is 0.0821 L atm/K mol, and T is absolute temperature in °K at which the osmotic shock was performed. Osmotic pressure online calculator hosted by Georgia State University was used for calculation of osmotic shock (http://hyperphysics.phy-astr.gsu.edu/hbase/kinetic/ospcal.html)

2.4. Chemical cell disruption

Tween 80, a detergent and ethyl acetate were mixed with 10% (w/v) of *A. halophila* cells separately at varying concentrations and were kept for 2 h at room temperature (RT 27 ± 2 °C).

2.5. Enzymatic cell disruption

All the enzyme mixes of trypsin (pH 8.0, 37 °C), protease (pH 9.5, 60 °C), papain (pH 7.0, 60 °C), pancreatin (pH 8.1, 37 °C) and lipase (pH 7.4, 37 °C) were prepared in 100 mM phosphate buffer