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Full Length Research Paper

Extracellular trehalose production by *Micrococcus lylae* and its enhancement by heat shock

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We examined the effects of heat (50℃), osmotic (3% NaCl), and solvent (3% ethanol) shock on the extracellular trehalose production by *Micrococcus lylae* KCTC 3517 in glucose-supplemented medium. Cultivation of *M. lylae* at 37℃ for 12 h followed by heat shock of exponen tially growing cells at 50℃ for 2 h at 12 h-intervals resulted in maximum trehalose production (1.72 mg/ml), which was 2-fold higher than that in untreated cells (0.85 mg/ml). Simple activated carbon column chromatography was then used to purify trehalose, which was eluted as major compound using 10-15% ethanol. Finally, the concentrated eluent contained moderately pure trehalose.

Key words: Trehalose, *Micrococcus lylae*, heat shock.

INTRODUCTION

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a non-reducing disaccharide that is widely distributed among microorganisms, plants, and animals (Elbein, 1974). Since trehalose is regarded as a reserve carbohydrate, its role under a variety of environmental stresses such as heat, freezing, desiccation, and osmotic pressure have been actively studied (Argüelles, 2000). These studies led to the broad use of trehalose in food processing industry, where it serves as a low calorie sweetener and a stabilizer by promoting increased water solubility and by lowering the freezing point (Roster, 1991). It is also used in the cosmetic and pharmaceutical industries, where it contributes to the stabilization of enzymes, vaccines, antibodies, and hormones (Paiva and Pane, 1996; Elbein et al., 2003).

Due to these potential applications, many studies have been conducted regarding efficient trehalose production. Extracting trehalose from thermally-treated yeast cells had been regarded as the general method used to produce trehalose, since trehalase (a hydrolytic enzyme of trehalose) can be suppressed by adjusting factors such as temperature, pH and ethanol concentration (Yoshikawa et al., 1994; Chuanbin et al., 1998). However, modifications to this strategy had been needed to overcome low product yields and high production costs. Therefore, various studies have been conducted to increase the trehalose production. For example, *Corynebacterium glutamicum* was metabolically engineered based on its trehalose biosynthetic pathway (Carpinelli et al., 2006) and the pathway of trehalose metabolism was characterized and engineered from *Propionibacterium freudenreichii* and *Lactococcus lactis* (Cardoso et al., 2007; Carvalho et al., 2011). Additionally, various approaches to trehalose production have been tried, particularly focusing on enzymatic conversion in microorganisms. The enzymatic production of trehalose from maltooligosaccharides has been described (Elbein et al., 2003; Maruta et al., 1995; Kato et al., 1996); however, it has not been actively used to study trehalose production from glucose, which could be a useful industrial starting material (Seto et al., 2004). Both

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Micrococcus sp. and *Deinococcus* sp. produce trehalose from glucose (Ahmad et al., 1980; Kizawa et al., 1995). However, of the *Micrococcus* strains, only a couple, *M. varians* and *M. luteus* have been studied in detail (Kizawa et al., 1995; Song et al., 1999). Therefore, the present study investigated the effects of various stresses on extracellular trehalose production by *M. lylae* cultured in glucose-supplemented medium.

MATERIALS AND METHODS

Microorganism and culture condition

Micrococcus lylae KCTC 3517 was obtained from the Korean Collection for Type Culture (KCTC), Republic of Korea. A seed culture of *M. lylae* was cultivated in Tryptic Soy Broth containing 1% glucose for 24 h at 37℃ and then transferred into a 500 ml flask containing 100 ml of main medium (2% glucose, 0.6% tryptone, 0.4% KH_2PO_4, 0.4% $MgSO_4 \cdot 7H_2O$, w/v) followed by cultivation at 37℃. To test the effects of various stressors on trehalose production, cells cultured for 12 or 24 h were subjected to heat shock at 50℃ for 2 h, osmotic shock with 3% NaCl, or solvent shock with 3% ethanol.

Trehalose assay

After cultivation, the cells were removed by centrifugation at 9,000 rpm for 15 min and the supernatants were used for an enzymatic assay to determine the capacity of trehalose production as previously described (Al-Bader et al., 2010). Briefly, the supernatants were treated with 0.22 U/ml of trehalase (Sigma), or left untreated as a control. The supernatants were then incubated at 42℃ for 6 h to allow the conversion of the produced trehalose to glucose. The concentration of released glucose was assayed using a glucose assay kit (Quantichrom) according to the manufacturer's instructions and then calculated from a pre-determined standard curve after subtracting the value of the untreated control. Finally, the trehalose content was determined from the calculated glucose concentration.

Purification of trehalose

Culture supernatant from a 2 L culture of heat shock-induced *M. lylae* was passed through filter paper (Whatman No. 6) to remove any residues and then loaded onto an activated carbon column (4.5×50 cm). The column was washed with water and subsequently eluted at a flow rate of 80 ml/h with a stepwise gradient of water/25% ethanol (v/v). Each eluent (100 ml) was concentrated *in vacuo* to 20 ml and subjected to an enzymatic assay as described above. The purity of trehalose was estimated by thin layer chromatography (TLC) as previously described (Seto et al., 2004). Briefly, the purified sample was applied to a silica-coated TLC plate (Merck) and then developed with a solvent mixture of *n*-butanol, acetic acid, and water (4:2:1, v/v/v). After the sample was developed, the plate was dried and visualized using an ethanol solution containing 20% H_2SO_4 (v/v).

RESULTS AND DISCUSSION

Preliminary experiment was carried out to compare with the amount of extracellular and intracellular trehalose from *M. lylae* KCTC 3517, exhibiting that the intracellular amount of trehalose (0.05 mg/ml) was too low compared to the extracellular production (0.79 mg/ml) without any stress. This result is strongly supported by the previous study indicating that most *Micrococcus* species including *M. lylae* produced the higher amount of trehalose extracellularly than intracellularly (Kizawa et al., 1995). Therefore we focused on just the extracellular trehalose production for further experiments. When *M. lylae* KCTC 3517 reached the stationary growth phase after 24 h-cultivation at 37℃, the strain was subjected to heat, osmotic, or solvent shock, and displayed a growth pattern similar to that observed under normal condition (no stress), even though the strain showed signs of inhibited cell growth. However, the amount of trehalose produced by *M. lylae* exposed to heat shock was 1.16 mg/ml, 30% higher than that by untreated cells or cells exposed to other types of stress after 48 h (data not shown). We also investigated trehalose production when the stress-starting point was changed to 12 h cultivation, when the cells were growing exponentially. Unlike shock induction during the stationary phase, the cell density of *M. lylae* under each shock condition decreased compared with that under normal condition. However, trehalose production (1.62 mg/ml) induced by heat shock increased remarkably, with a yield approximately twice that obtained under normal condition (0.79 mg/ml) after a 36 h-cultivation. On the other hand, osmotic and solvent shock increased trehalose production by 1.5- and 1.3-fold, respectively (data not shown). These results suggest that exposing exponentially growing *M. lylae* to heat shock induces trehalose biosynthesis, whereas osmotic and solvent shock does not relatively influence the trehalose production. In fact, the role of trehalose as a stress-protective molecule under adverse conditions has been well studied, although most studies focused on yeast and other fungi. *Saccharomyces cerevisiae* accumulates trehalose after heat shock at 37℃, but not after osmotic shock by 300 mM NaCl, which is consistent with our results (Lewis et al., 1995). *Schizosaccharomyces pombe* and *Aspergillus fumigates* also accumulate trehalose in response to heat exposure to 40 and 50℃, respectively (Virgilio et al., 1990; Al-Bader et al., 2010). It should be noted that trehalose produced by heat shock-treated *Schizo pombe* rapidly degraded after shifting the cultivation temperature back to 27℃. This tendency was also observed in the present study when the cultures were shifted back down to 37℃ after a 2 h-heat shock at 50℃. This might be due to increased trehalase activity after recovery from heat shock (Virgilio et al., 1990).

Our results, demonstrating the induction of trehalose production after heat shock and its decrease after recovery from heat shock, strongly encouraged us to investigate the effects of heat shock intervals on trehalose production. After cultivation for 12 or 24 h at 37℃, *M. lylae* cells were heat shocked at 50℃ for 2 h at 12 or 24 h-intervals. As shown in Figure 1, heat shock induced higher levels of trehalose production overall than

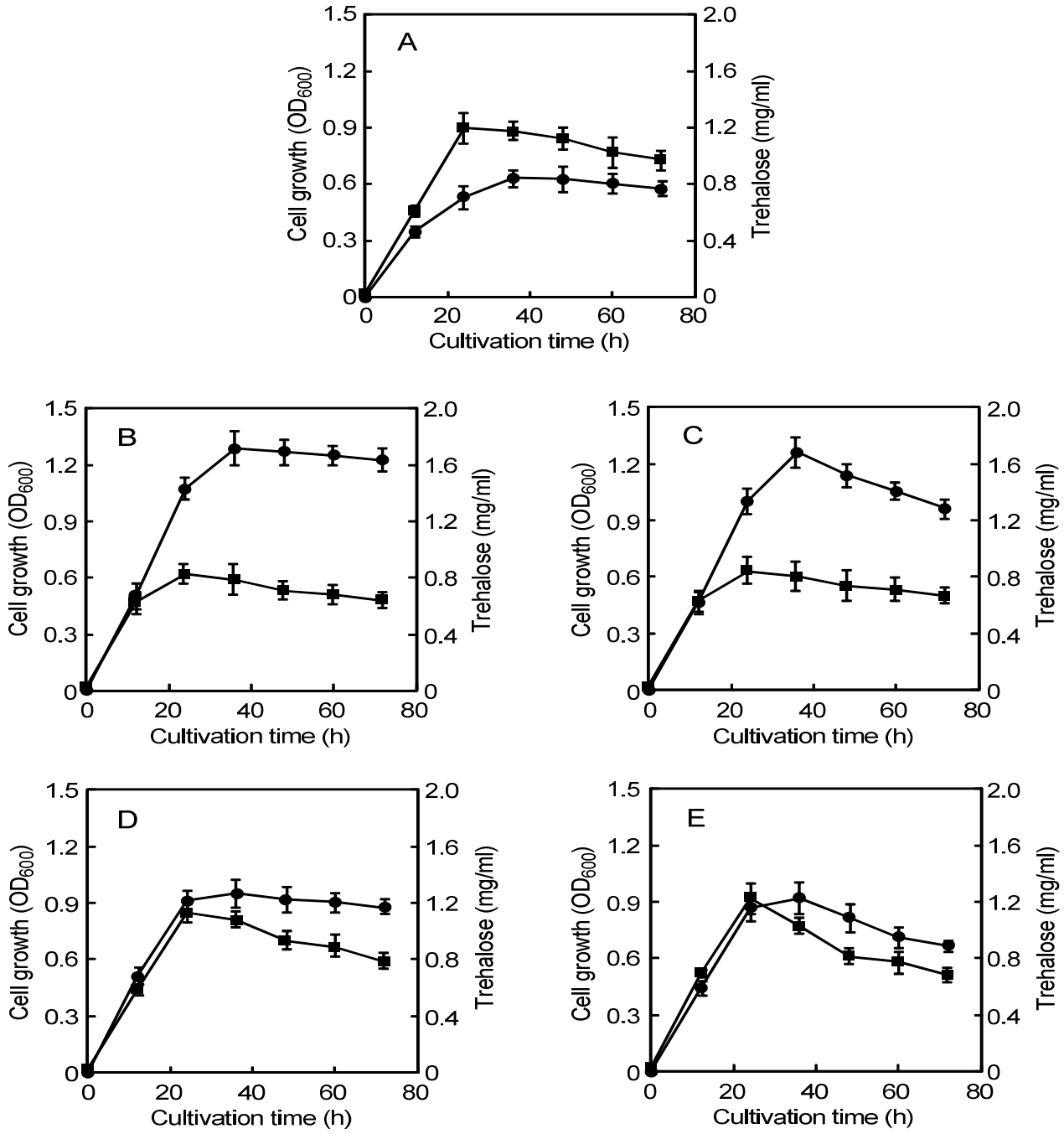

Figure 1. The effects of different heat shock intervals on cell growth (squares) and trehalose production (circles) by *M. lylae*. After 12 h (B, C) or 24 h cultivation (D, E) at 37℃, *M. lylae* was subjected to heat shock at 50℃ for 2 h with 12 h- (B, D) or 24 h-intervals (C, E). Cell growth was determined by monitoring the optical density at 600 nm (OD_{600}). Normal condition (A) represents non-stressed *M. lylae*. Each value represents the mean of triplicate measurements with standard deviation.

normal condition (0.85 mg/ml). There was no significant difference in the maximum trehalose content between 12 h (1.72 mg/ml) and 24 h-intervals (1.69 mg/ml) when the same stress-starting point occurred after 12 h of cultivation or between 12 h (1.27 mg/ml) and 24 h-intervals (1.23 mg/ml) after 24 h of cultivation. However, the degradation of trehalose seemed to be suppressed by heat shock at 12 h-intervals in comparison with 24 h-intervals, indicating that short-term heat shock moderates trehalase activity after heat shock (Hottiger et al., 1987;

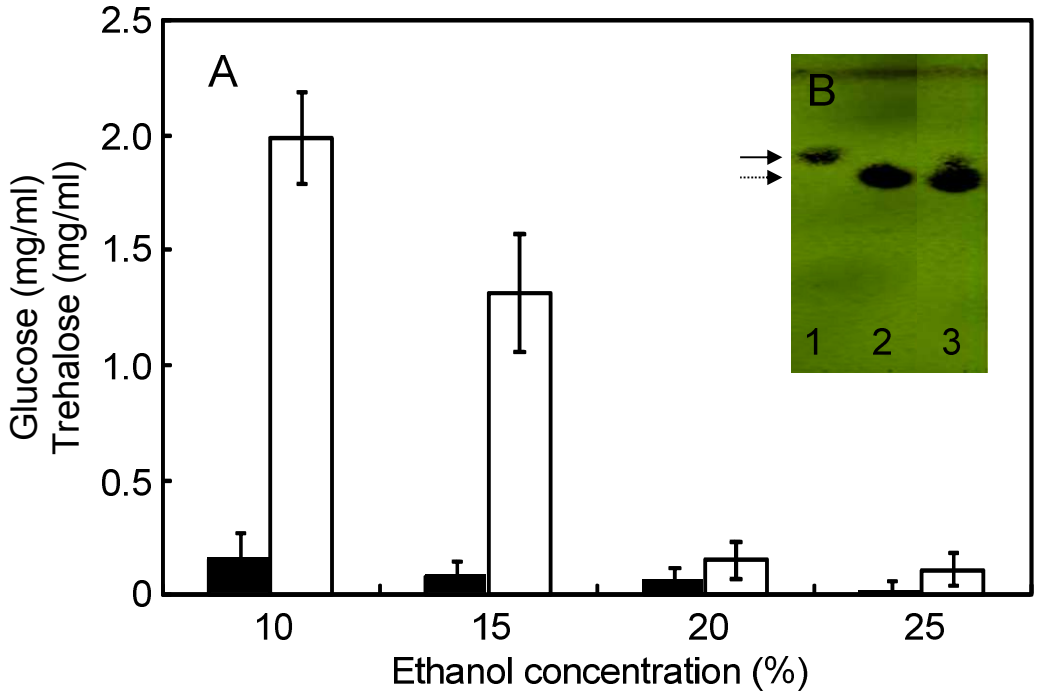

Figure 2. Separation of trehalose (unfilled bars) from glucose (filled bars) in the culture broth from heat shock-induced *M. lylae* using activated carbon column chromatography (A) and confirmation of trehalose purity by TLC analysis (B). Lanes 1 and 2 indicate authentic glucose and trehalose standards, respectively. Lane 3 showed trehalose purified from heat shock-induced *M. lylae*. Solid and dashed arrows represent spots corresponding to glucose and trehalose, respectively. Each value represents the mean of triplicate measurements with standard deviation.

Virgilio et al., 1990).

We used activated carbon column chromatography for the simple purification of trehalose. The samples eluted in 5% ethanol contained only glucose, whereas trehalose was eluted as major compound when 10-15% ethanol was used, although a small amount of glucose was also eluted (Figure 2A). The fractions containing trehalose were collected, concentrated *in vacuo*, and then freeze-dried to produce a moderately pure trehalose preparation. Thin layer chromatography (TLC) analysis of the purified sample revealed the presence of a marked spot corresponding to trehalose, although the monosaccharide glucose spot did not disappear completely, suggesting that the trehalose produced by *M. lylae* was quite pure after simple activated carbon chromatography (Figure 2B).

In conclusion, we showed that *M. lylae* KCTC 3517 produced extracellular trehalose and its biosynthesis and secretion were induced by exposing exponentially-growing cells to repeated stimulus (12 h-intervals) of heat shock at 50℃ for 2 h. Increased trehalose producti on in response to heat shock facilitates the stabilization of whole cells, preventing the cytosol from being impaired by heat shock or dehydration due to exposure to high temperatures (Hottiger et al., 1987; Paik et al., 2005). As far as we know, few studies have examined extracellular trehalose production from microbial cultivation from glucose (Kizawa et al., 1995; Seto et al., 2004) and increased trehalose production by *M. lylae* induced by various stressors; therefore, the results of this study may contribute to the development of a useful trehalose production system. We also used a simple purification system incorporating an activated carbon column to easily separate trehalose from glucose. Thus, *M. lylae* has the potential to produce trehalose for commercial application in the food and cosmetic industries if the trehalose yield can be increased by a combination of heat shock and optimization of the cultivation and purification conditions (Lewis et al., 1995; Wang et al., 2008).

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