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Screening bacterial strains for production of maltooligosyl trehalose trehalohydrolase and maltooligosyl trehalose synthase

Sàng lọc các chủng vi khuẩn có khả năng sản xuất maltooligosyl trehalose trehalohydrolase và maltooligosyl trehalose synthase

Research article

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Maltooligosyl trehalose synthase (MTSase, EC 5.4.99.15) catalyzes the synthesis of maltooligosyl trehalose by converting the of α (1 → 4) glucosidic linkages on the reducing ends of maltooligosaccharides to α (1 →1) glucosidic linkages. Maltooligosyl trehalose trehalohydrolase (MTHase, EC 3.2.1.141) catalyzes the release of trehalose by cleaving the α-1.4-glucosidic linkage next to the α-1.1-linked terminal disaccharide of maltooligosyl trehalose. Trehalose was synthesized from starch by the cooperative action of these two enzymes. Trehalose is of great interest in many industrial fields. Until now, many studies have been performed to develop effective methods of trehalose production. This research focused on screening strains bacteria were able to produce of trehalose from starch which is novel and economic method for trehalose production. We selected two strains that had MTSase and MTSase strong activity from ten strains that were isolated in Vietnam.

Maltooligosyl trehalose synthase (MTSase, EC 5.4.99.15) xúc tác cho phản ứng phân hủy maltooligosaccharide thành maltooligosyl trehalose bằng chuyển đổi glycosyl hóa nội phân tử sau đó maltooligosyl trehalose trehalohydrolase (MTHase, EC 3.2.1.141) thủy phân đặc hiệu maltooligosyl trehalose thành trehalose. Phương pháp sản xuất trehalose từ tinh bột bằng cách sử dụng MTSase và MTHase có tiềm năng ứng dụng trên quy mô lớn với một chi phí khả thi để có thể thương mại hóa sử dụng cho ngành công nghiệp thực phẩm. Trong nghiên cứu này chúng tôi sàng lọc khả năng sản xuất trehalose của 10 chủng vi khuẩn từ đó chọn ra hai chủng có hoạt tính MTSase và MTHase xúc tác cho phản ứng tạo trehalose từ tinh bột tan.

Keywords: Maltooligosyltrehalose synthase, Maltooligosyltrehalose trehalohydrolase, Trehalose

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1. Introduction

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranose) is a nonreducing diglucoside found in various organisms, including bacteria, algae, fungi, yeasts, insects, and some plants [1]. From the wide variety of species that have been shown to contain trehalose it seems likely that trehalose may be present in many other organisms. Interestingly, trehalose has been isolated from all species of insects examined, is the principal sugar (approximately 80–90%) found in the hemolymph, and can constitute about 20% of all carbohydrates during specific stages of insect development [2]. Trehalose was reported to serve as a protective agent and/or carbohydrate reserve under such conditions as drought, heat, salinity, and oxidative stresses, owing to its novel structure of α-D-glucopyranosyl-[1,1]-α-D-glucopyranose [3]. It is significant for the survival of microorganisms exposed to extreme environmental stress.

Trehalose is of great interest in many industrial fields [4]. It has greater stability and less sweetness (45% that of sucrose) than sucrose. It also shows a moderate glycemic index with a low insulinemic response. In addition, other functional properties such as low cariogenicity, low hygroscopicity, high freezing -point depression, high glass transition temperature, and protein protection properties make it an important novel ingredient in the food industry. Trehalose is known to have a high water retention capacity and thus has been used as a moisturizer in the cosmetics industry. Furthermore, it is used for the lyoprotection of therapeutic proteins, especially for parenteral administration in pharmaceutical applications. Until now, many studies have been performed to develop effective methods of trehalose production.

Biosynthetic pathways for the production of trehalose are found in various organisms. In microorganisms such as *Escherichia coli* and yeast trehalose-6-phosphate synthase (T-6-P) works by forming T-6-P from UDP-glucose and glucose-6-phosphate, which is cleaved into trehalose and phosphate by T-6-P phosphatase [5]. In some bacteria, the biosynthesis of trehalose is mediated by maltooligosyltrehalose synthase (MTSase) and maltooligosyl trehalose trehalohydrolase (MTHase). MTS frist catalyzes the transglycosylation of the terminal maltosyl residue into a trehalosyl residue, producing a nonreducing maltooligosyltrehalose. MTHase then cleaves the α-1.4-glucosidic linkage next to the α-1.1-linked terminal disaccharide of maltooligosyltrehalose, thus releasing a trehalose molecule and regenerating a substrate for MTSase [6, 7]. These two enzymes and their corresponding genes have been isolated from *Arthrobacter* sp [6, 7]. Then, many sources of bacteria having MTHase and MTSase were isolated as *Arthrobacter ramosus* S34 [8], *Corynebacterium glutamicum* [9], *Deinococcus radiodurans* [10], *Rhizobium sp*. M-11 [11], *Sulfolobus solfataricus* MT4 [12], *Sulfolobus solfataricus* KM1 [13], *Sulfolobus acidocaldarius* ATCC 33909 [14], and *Sulfolobus shibatae* DMS 5389 [15].

Usually trehalose is extracted from yeast cells. However this method is unsuitable for industrial production due to its low yield and high cost. In some cases, synthesizing trehalose by microbial enzymes has been used in industrial production of trehalose in Japan. Using the enzymes to produce trehalose from starch has economic advantage because starch is a relatively inexpensive substrate; therefore, could lower the production cost. In this study, we screening bacterial strains were able to produce of trehalose and identify characteristic of these strains.

2. Materials and methods

2.1. Materials

Chemicals: Trehalose, silica gel plate (Merck, USA), API 20E kit (bioMerieux, USA)

Bacterial strains

Nine strains from Laboratory of Enzyme Biotechnology (Institute of Biotechnology) and one strain from Vietnam Type Culture Collection (VTCC) used are listed in Table 1.

Table1. List of strains used for this study

N_o	Symbol	N_o	Symbol
1	2_2	6	$ĐN_4$
2	5_6 CT	7	*P. putida* VTCC - B2263
3	$ĐT_1$	8	$DĐ_1$
4	M_5	9	HK_1
5	9_1	10	$TBXL_347$

2.2. Method

2.2.1 Growth media and growth conditions

The growth medium used was Luria-Bertani broth (g/L): 10 peptone, 5 yeast extract, 10 NaCl). The osmotic strength of the medium was increased by the addition of 1% (w/v) NaCl or 2% (w/v) glucose. The bacteria were grown at 37°C with agitation at 220 rpm.

2.2.2 Screening process for trehalose-producing bacteria

Bacteria were cultivated in 5 ml of LB medium at 37°C with agitation at 220 rpm. Five hundred µl of the overnight culture were transferred into 50 ml of LB medium containing 1% (w/v) NaCl or 2% (w/v) glucose. The culture was cultivated at 37°C with agitation at 220 rpm for 24 hours. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C.

2.2.3 Measurement of intracellular trehalose

Trehalose was determined according to the method of Purvis 2005 [16]. Sufficient culture volume was harvested (12.500 rpm, 25°C) to provide 2.0 mg dry cell weight. Cells were permeabilized with 50% methanol and extracted for 30 min on ice. The mixture was mixed

vigorously and centrifuged at 12,500 rpm for 1 min. The supernatant was assayed for trehalose by thin-layer chromatography.

2.2.4 Assay for Trehalose-Forming Activity

Cell concentrates were broken by using an Ultrasonic Cell Disruptor in a mini-mum amount of phosphate buffer (20 mM, pH 7.0) and centrifuged and the supernatant was used for trehalose-forming activity by adding 1% soluble starch (substrate). Trehalose synthesis was assayed as follows: The reaction mixture (5 mL) containing substrate (2.5 mL) and 20 mM sodium phosphate buffer (2 mL, pH 7.0) plus supernatant (0.5 mL) was incubated at 37°C for up to 24 h and was terminated by heating the reaction mixture at 95°C for 5 min. Products of the enzyme reactions were analyzed by thin-layer chromatography (TLC)

2.2.5 Qualitative Analysis of Trehalose by TLC

A standard of trehalose (50 mM; Merck) was prepared. Samples (5 ml) were spotted onto a silica gel plate (Merck, Germany) along with the trehalose standard and developed in a closed chamber with the n-butanol–ethanol–water (5:3:2 v/v) mobile phase. The spots were detected by spraying with 20% sulfuric acid and 5% ethanol followed by heating at 130°C as described by Kim et al [17]

2.2.6 Characteristic of selected strains

Biochemical and physical tests were performed following manufacturer's instructions of kit API 20E.

3. Results and discussion

3.1. Screening for trehalose production

The effects of increasing trehalose production were examined during growth in response to osmotic stress with sugars and additional inorganic salts. Increasing the production of trehalose resulted in increased growth under osmotic stress [16]. So to investigate the ability to produce of trehalose biosynthesis, ten strains were cultured under sugar and salt stress conditions. After strains had been cultivated in stress medium, the extract cell was analyzed. The intracellular trehalose of these strains were identified by TLC.

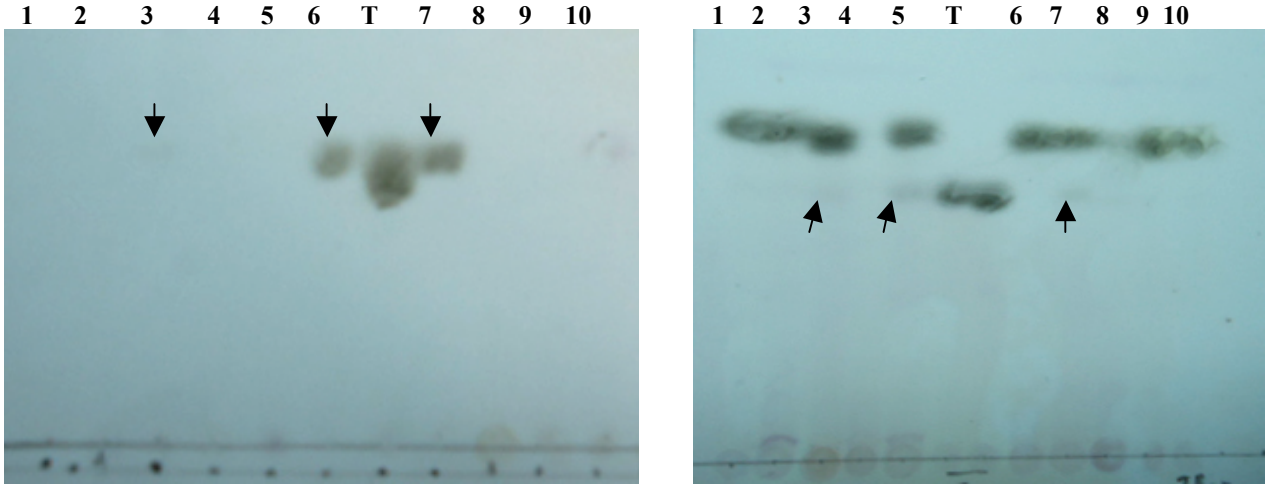


Figure 1. Qualitative analysis of trehalose-producing from screening strains by TLC (A) salt stress; (B) sugar stress 1: 2_2; 2; 5_6CT; 3: ĐT$_1$; 4: M$_5$; 5: 9_1; 6: ĐN$_4$; 7: P.putida; 8: DĐ$_1$; 9: HK$_1$; 10: TBXL$_3$47. T: Trehalose (Sigma)

TLC analysis of cell extraction different strains grown on salt and sugars stress showed that: at salt stress condition ĐT$_1$; ĐN$_4$ and *P. putida* strains had one band corresponding to trehalose, may be intracellular trehalose were formed (Fig 1A). However, at sugar stress condition ĐT$_1$; 9_1 and *P. puti*da had one band corresponding to trehalose (Fig 1B). So we found that different types of stress had different impact on the formation of trehalose. Our finding is similar to previously reported. Asthana et al showed that trehalose level were different at salt-stressed cells with different NaCl concentrations, and differences between the protein levels were because of the salt stress. This mean that under different stress, protein levels changes so that that the level of trehalose production will be different [18, 19]. Therefore, we selected four strains ĐT$_1$; ĐN$_4$, 9_1 and *P. putida* for secondary screening through MTSase and MTHase activity assay.

3.2. MTSase and MTHase activity

There are four pathways for synthesis trehalose in bacteria. The most common pathway involves trehalose-phosphate synthase and trehalose-6-phosphate phosphatase from uridine diphosphoglucose or guanosine diphosphate glucose [20, 21]. Trehalose may also be synthesized directly from maltose via trehalose synthase [3, 4]. A third pathway found in several bacteria converts the terminal unit of a glucose polymer such as starch or maltooligosaccarid to trehalose via MTSase and MTSase [5, 11]. Formally, trehalose phosphorylase forming glucose and glucose-1-P from trehalose may also be regarded as a trehalose-synthesizing enzyme because the

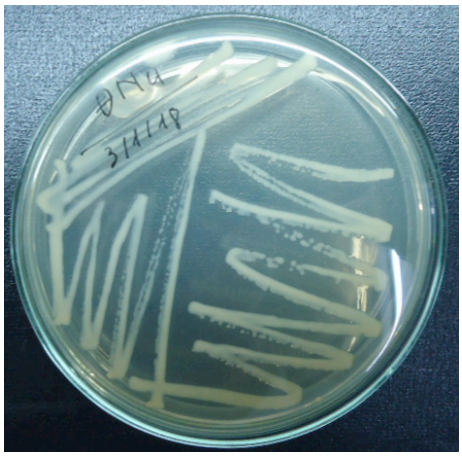
reaction is reversible at least in vitro [22, 23]. Thus, there were only one pathway that converts starch into trehalose. It is through MTHase and MTSase.

The MTSase and MTHase activity of 4 selected strains was screened by using trehalose-forming activity assay with starch as substrate. TLC analysis of product generated by the enzyme on starch was performed. Results are reported in Fig. 2. Negative controls, which did not contain cell lysate, showed no alteration of the substrate during incubation (S). After 24 hours of incubation, the presence of trehalose producing were detected on reaction of ĐT1, $ĐN_4$ and *P.putida* cell lysate with starch, In particular, the trehalose producing from reaction of $ĐN_4$ and *P. putida* cell lysate and starch is the most obvious. However, it is not the only product of the reaction, because maltotetraose, maltotriosyl trehalose and glucose can be visualized (Fig. 2). We did not observe any product of 9_1 sample. This means that the trehalose synthesis enzyme existed in 9_1 cell lysate is not MTHase and MTSase, so it is impossible to convert starch into trehalose. Strain $ĐN_4$ and *P. putida* were selected for further study.

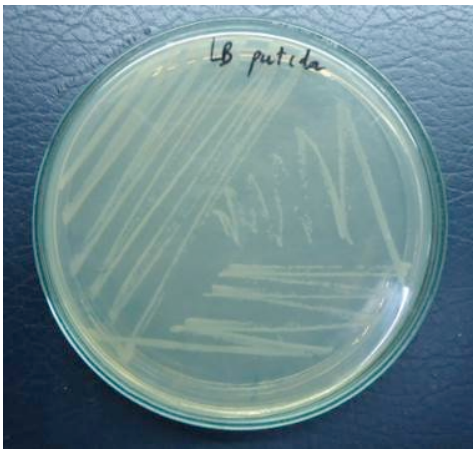
Figure 2. Visualization of MTSase and MTHase catalysis reaction products by TLC (3: ĐT1 ; 5: 91 ; 6: ĐN4; 7: P. putida; S: starch, T: Trehalose)

3.3. Characteristic of selected strains

Two selected strain were test biochemical characteristic



ĐN4



P. putida

Figure 3. Morphorlogy clony of $ĐN_4$ and *P. putida*

Clony shape of two strains are circular. Colors of $ĐN_4$ is white and colors of *P. putida* is yellowish white (Fig 3). $ĐN_4$ has urease activity, *P. putida* has arginine dihydrolase activity and capable of producing glucose, melibiose, arabinose (Table 2).

Table 2. Physiological and biochemical characteristics of ĐN$_4$ and *P. putida*

Characteristics	ĐN$_4$	*P. putida*
ONPG: test for β-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside	N	N
ADH: decarboxylation of the amino acid arginine by arginine dihydrolase	N	P
LDC: decarboxylations of the amino acid lysine by lysine decarboxylase	N	N
ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase	N	N
CIT: utilization of citrate as only carbon source	N	N
H_2S: production of hydrogen sulfide	N	N
URE: test for the enzyme urease	P	N
GEL: test for the production of the enzyme gelatinase which liquefies gelatin	N	N
GLU: fermentation of glucose	N	P
MAN: fermentation of mannose	N	N
INO: fermentation of inositol	N	N
SOR: fermentation of sorbitol	N	N
RHA: fermentation of rhamnose	N	N
SAC: fermentation of sucrose	N	N
MEL: fermentation of melibiose	N	P
AMY: fermentation of amygdalin	N	N
ARA: fermentation of arabinose	N	P

N**:** Negative, P: positive

4. Conclusion

In a group of bacteria, trehalose is synthesized by MTSase and MTHase from starch. Our study found four strains capable of producing intracellular trehalose under osmotic stress conditions, of which two have catalytic enzymes that convert starch into trehalose were ĐN$_4$ and *P. putida* VTCC- B2263. These strains could be of potential use for manufacturing trehalose on an industrial scale. But the use of microbes for industrial purposes demands a new breed of organisms that must be engineered for optimal growth under specific physical and chemical parameters.

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