ACTIVITY AND PRIMARY CHARACTERIZATION OF ENZYME FROM *THERMUS RUBER* CELLS CATALYZING CONVERSION OF MALTOSE INTO TREHALOSE

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ABSTRACT

Thermophilic bacteria Thermus ruber *produces enzyme, which catalyzes the conversion of maltose into trehalose. The specific activity of the cell-free extract from this bacteria growing without inducers was 0.028 U/mg protein and it was increased to up to 0.086 U/mg in the presence of 0.5% of maltose in the culture broth. The maximum degree of maltose conversion of about 90% was attained at 10% substrate concentration. The enzyme from* Thermus ruber *does not catalyze formation of trehalose from maltotetraose and maltopentaose. The optimum temperature for the enzyme activity was 65C. A maximum activity of the maltose conversion was performed at pH 6.5. The highest enzyme activity was achieved during cell cultivation at 55C on a media composed from 0.5% of peptone, 0.1% yeast extract and 0.5% of maltose or starch.*

PRACTICAL APPLICATIONS

Trehalose is a chemically stable nonreducing disaccharide which can be used in food, cosmetics, medical and biotechnological industries. Extraction of this carbohydrate from yeast cells or other natural sources is unsuitable for trehalose production because of low process yield and high cost. Thus, the enzymatic methods of trehalose production are developed. In the current study the thermophilic bacteria *Thermus ruber* has been examined as a new source of the enzyme catalyzing conversion of maltose into trehalose.

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INTRODUCTION

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a chemically stable, nonreducing disaccharide containing two D-glucose residues bound in an α,α-1,1-glucosidic linkage. The unique properties of trehalose suggest a wide range of application in the food, cosmetics, medical and biotechnological industries, as well as for preservation of bacteria and yeast cultures, stabilization of vaccines, antibodies, pharmaceutical preparations and organs for transplantation (Schiraldi *et al.* 2002). This carbohydrate is not browning, and will not hydrolyze at low pH or elevated temperature (Crowe and Crowe 2000). Trehalose can form hydrogen bonds with protein and lipid molecules, resulting in improved stability of cell structures during freezing, desiccation or heating (Crowe *et al.* 1988; Roser 1991).

Trehalose may be used as a multifunctional ingredient of many food products including beverages, chocolate and sugar confectionery, bakery, dairy and fruit products, and for the preservation of surimi and other frozen foods. The amorphous, low-hygroscopic glass of trehalose has been reported to be effective for protection of tissues against damage caused by ice-crystal formation and may hold protein molecules in a form that permits them to return to native structure (Crowe and Crowe 2000). In addition, trehalose glasses are almost not permeable to hydrophobic, aromatic esters and this property allows for conservation of the natural flavor of the dried vegetables and fruits (Richards *et al.* 2002). The replacement of other sugars with trehalose limits the Maillard reactions contributed to color and flavor changes of the food products, reduces its moisture sensitivity and may help to maintain the texture of frozen foods. Furthermore, the mild sweetness of trehalose can enhance a natural taste in food systems (Roser 1991; Portmann and Birch 1995; Singer and Lindquist 1998).

The method of trehalose extraction from yeast or other natural sources is unsuitable for industrial production because of its low yield and high cost. Thus, the trehalose-synthesizing enzyme systems that originated from a few microorganisms have been investigated (Di Lernia *et al.* 1998). For trehalose synthesis in bacteria or archaea, three different pathways are exploited. The first one is a two-step process using a successive action of glucose(maltose) phosphorylase and trehalose phosphorylase. Some microorganisms can produce trehalose from maltodextrins in two sequential steps catalyzed by maltooligosyl-trehalose synthase (MTSase) and maltooligosyl-trehalose trehalohydrolase (MTHase) (Koen *et al.* 2000). It was also found that *Pimelobacter* sp. and some other bacteria produce trehalose synthase (maltose-α-D- glucosyl transferase, EC 5.4.99.16) (TreS) that catalyzes the reversible interconversion of maltose (glucosyl-α-1,4-glucose) and trehalose (glucosyl-α,α-1,1-glucose) (Nishimoto *et al.* 1996a,b; Ohguchi *et al.* 1997; Ma *et al.*

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2006). This enzyme can be used for production of trehalose from maltose syrups. In the current study, a thermophilic bacteria, *Thermus ruber*, was examined as a new source of thermostable enzyme catalyzing conversion of maltose into trehalose. The use of heat-resistant enzymes allows us to achieve a higher conversion yield of maltose and limits the risk of microbial contamination of immobilized enzyme system.

MATERIALS AND METHODS

Microorganism and Cultivation

T. ruber (DSM 1279) was cultivated under aerobic conditions on liquid media (pH 8.0) containing 0.5% of peptone bio-Trypcase (bioMerieux, Craponne, France) and 0.1% of yeast extract (Difco Laboratories, Lawrence, KS). The growth media were sterilized for 30 min at 121C and supplemented with different amounts of sodium chloride or carbohydrates. The inoculum was prepared by incubation (48 h, 55C) of lyophilized *T. ruber* cells with 5 mL of the growth medium. The cultures were then incubated at 50, 55, 60 or 65C in Erlenmeyer flasks with a working volume of 1 liter with the agitation rate of 160 rpm. After 24 or 48 h of growth without additional aeration, the cells were harvested by centrifugation at 9,000× g for 15 min, washed with 5 mM Na_2HPO_4 solution, centrifuged and stored at −18C until use. Frozen cells (10 g) were disrupted for 15 min in a refrigerated mortar with 20 g of Alumina A-5 (Sigma, St. Louis, MO) and 70 mL of 0.01 M phosphate buffer (pH 6.2) containing 1 μmol dithiotreitol per 1 mL of the buffer (which was gradually added during extraction). The cellular extract obtained after centrifugation (9,000× g for 15 min) was used (as a crude enzyme) to study the activity and the best conditions of maltose conversion.

Enzyme Assay

The activity of the substrate conversion was assayed by measuring trehalose produced from maltose. The assays were initiated by the addition of 5 mL of crude enzyme preparation to 10 mL of 30 mM maltose solution in 0.1 M phosphate citrate buffer (pH 6.5). The reaction at 65C was terminated after the desired time by cooling in ice. The products of maltose conversion were identified by high-performance liquid chromatography (HPLC) using APS-2 Hypersil column (Thermo Electron Corporation) and refractive index detector (La Chrom L-7490, Merck, Darmstadt, Germany). The samples purified by thermal precipitation (110C) of the enzyme, adsorption on charcoal, centrifugation (9,000× g) for 15 min and filtration on 0.2 μm Puradisc ™ filter (Whatman), were passed through a column using acetonitril/water (3:1 v/v) as

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the mobile phase at a flow rate of 0.6 mL/min. The column temperature was 25C. Trehalose, maltose and glucose were used as standards at concentration of 10 mg/mL.

One unit of the enzyme activity (U) is defined as the amount of the enzyme required for production of 1 μmol of trehalose per minute under described assay conditions. Specific activity is the enzyme units per milligram of protein. Protein was determined by Bradford (1976) using bovine serum albumin as a standard. The relative enzyme activity (%) was defined as the percentage of the enzyme activity in the control.

Some Properties and Thermostability of the Examined Enzyme

The effect of pH on the enzyme activity was determined at 65C using 30 mM maltose solution in 0.1 M phosphate citrate buffers in the pH range of 5.0–8.0 adjusted at 65C. The temperature-dependence on enzyme activity was assayed in the range of 50–85C. Thermal stability of the cellular extract of *T. ruber* was determined by 1 h incubation of the samples in sealed tubes at temperatures between 40 and 80C. The pH of the samples was adjusted to the value of 6.0 or 7.0 using 0.1 M phosphate citrate buffers. After incubation, the samples were cooled in ice and assayed for remaining activity at 65C and pH 6.5.

Determination of the Enzyme Activity toward Starch and Some Oligosaccharides

The assays were initiated by the addition of 5 mL of crude enzyme to 10 mL 1% solutions of starch, amylose, maltopentaose or maltotetraose (Sigma) in 0.1 M phosphate citrate buffer (pH 6.5). After 24 h of reaction, 5 μL of aliquot were removed and subjected to thin-layer chromatography on a plate (10 × 20 cm) of silica gel G-60 (Merck). The chromatograms were developed two times in a mobile phase system composed of butanol, pyridine and water (6:4:3, v/v/v). The sugar spots were visualized by spraying with the reagent consisting of sulfuric acid and methanol (1:9, v/v), followed by incubation at 105C for 30 min. As standards, 0.1% solutions of glucose, maltose, trehalose, maltotetraose and maltopentaose were used.

RESULTS AND DISCUSSION

The HPLC analysis show that the product obtained after action of the proteins extracted from *T. ruber* cells on maltose consists of glucose and trehalose (Fig. 1). It suggests that *T. ruber*, like some other thermophilic bacteria belonging to the genus *Thermus*, also produces trehalose synthase.

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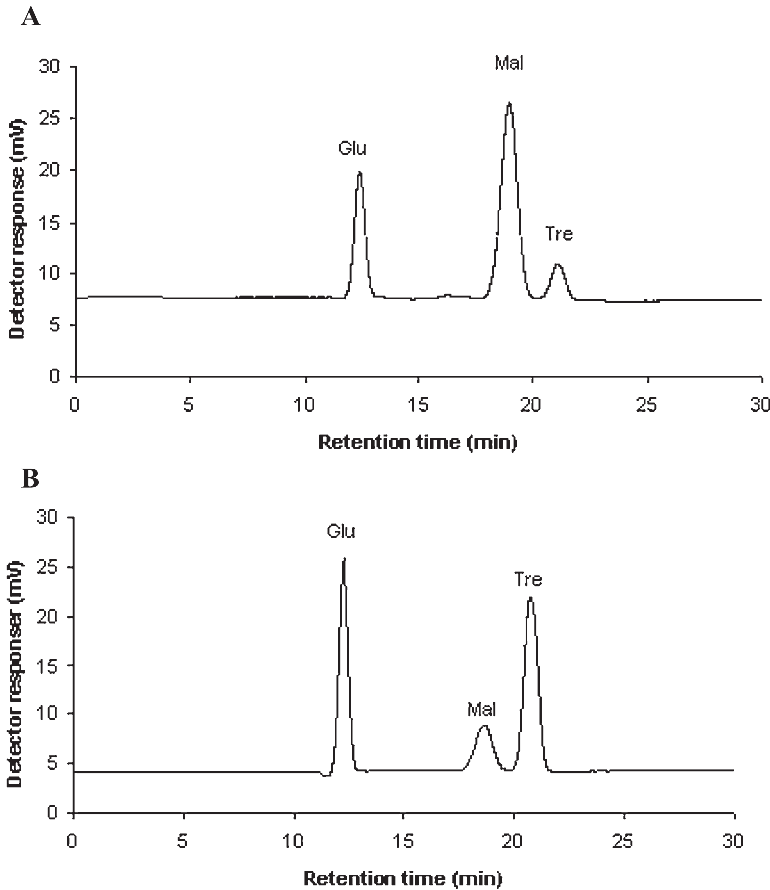

FIG. 1. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF THE PRODUCTS: OBTAINED AFTER 15 min (A) AND 120 min (B)
Maltose conversion catalyzed at 65C in phosphate citrate buffer (pH 6.5) by cell-free extract of *Thermus ruber* cells. The shifts represent glucose (Glu), trehalose (Tre) and residual maltose (Mal).

However, the gene sequence of the target bacterium is not known and trehalose can also be produced by other enzyme systems. This phenomenon was observed in the case of some genera belonging to cyanobacteria that can exhibit different ways of trehalose production (Asthana *et al.* 2005). The conclusion that *T. ruber* produces trehalose synthase is strongly supported by a similarity of the properties and substrate specificity of the enzyme from this bacteria to the trehalose synthases described in the literature (Nishimoto *et al.* 1996a,b). The accumulation of glucose during maltose conversion is similar to

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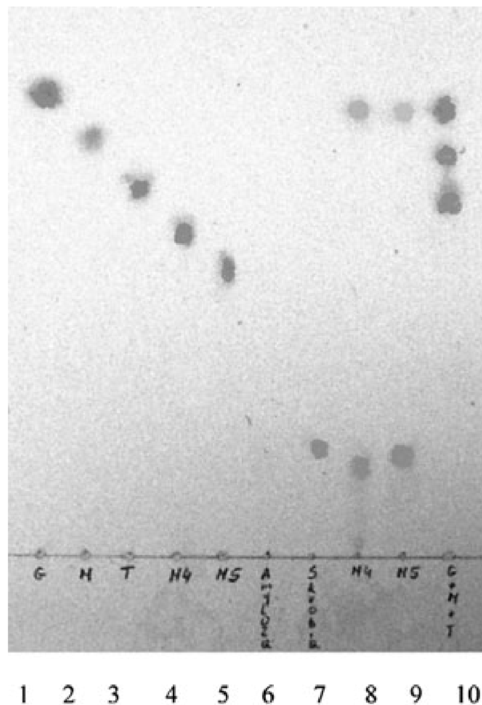

FIG. 2. THIN-LAYER CHROMATOGRAPHY OF THE PRODUCTS OBTAINED AFTER ACTION (65C, 24 h) OF THE ENZYME FROM *THERMUS RUBER* ON 1% SOLUTIONS OF AMYLOSE (LANE 6), STARCH (LANE 7), MALTOTETRAOSE (LANE 8) OR MALTOPENTAOSE (LANE 9)

Standards: 1, glucose; 2, maltose; 3, trehalose; 4, maltotetraose; 5, maltopentaose; 10, glucose, maltose and trehalose mixture.

that for trehalose sythases from *Thermus aquaticus*, *Pimelobacter* sp. R48 and *Pseudomonas* sp. F1, which also show a weak hydrolytic activity (Nishimoto *et al.* 1996a,b; Ohguchi *et al.* 1997). Furthermore, maltose-converting enzyme from *T. ruber* does not catalyze formation of trehalose from maltotetraose and maltopentaose (Fig. 2). It also suggests that the enzyme from *T. ruber* is a true trehalose synthase, because the system of MTSase and MTHase existing in *Sulfolobus shibatae*, *Arthrobacter* sp. Q36 and several other microorganisms can produce trehalose from starch and low-molecular weight oligosaccharides (Di Lernia *et al.* 1998). However, the investigated enzyme was completely inactive on starch and amylose. The observed formation of glucose from maltotetraose and maltopentaose is a result of residual α-glucosidase activity in a cell-free extract of *T. ruber* proteins. When glucose was incubated with the enzyme, none of the products was detected by HPLC (data not shown).

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TABLE 1.
EFFECT OF TEMPERATURE AND TIME OF CULTIVATION ON YIELD OF *THERMUS RUBER* CELLS

Growth time (h)	Wet cells (g/L growth medium) at temperature (C)			
	50	55	60	65
24	4.32 ± 0.17	4.48 ± 0.21	3.49 ± 0.25	2.70 ± 0.40
48	5.71 ± 0.08	5.95 ± 0.28	3.76 ± 0.23	2.78 ± 0.56

The results are mean values of data from four cultivations on a media containing peptone and yeast extract ± standard deviations.

TABLE 2.
EFFECT OF TEMPERATURE AND CULTIVATION TIME ON ACTIVITY AND PRODUCTIVITY OF TREHALOSE PRODUCING ENZYME IN *THERMUS RUBER* CELLS

Temperature (C)	Growth time (h)	Total activity (U/g of cells)	Specific activity (U/mg protein)
50	24	0.311 ± 0.041	0.016 ± 0.003
	48	0.307 ± 0.042	0.017 ± 0.002
55	24	0.659 ± 0.032	0.020 ± 0.001
	48	0.604 ± 0.065	0.028 ± 0.002
60	24	–	0.028 ± 0.008
	48	0.366 ± 0.046	0.017 ± 0.004
65	24	0.473 ± 0.039	0.016 ± 0.002
	48	0.358 ± 0.001	0.017 ± 0.001

The results are mean values of data from four cultivations ± standard deviations.

Production of trehalose was influenced by temperature and time of *T. ruber* cultivation. The bacteria grows well at 55C on a liquid medium containing peptone and yeast extract (Table 1), and the final yield of the wet cells after 48 h of cultivation was about 5.95 g per liter of the medium. However, a large decrease of the wet biomass yield up to 3.76 and 2.78 g/L after 48 h of bacteria growth was observed at temperatures 60 and 65C, respectively (Table 1). The highest enzyme productivity was achieved at an optimal temperature of *T. ruber* growth. It shows that the total activity of the enzyme in the cells growing at 55C was intensified by about 50% of the value obtained during cultivation of the bacteria at 50C and 60C (Table 2). *T. ruber* was cultivated in the presence of carbohydrates to assess their effect on the production of trehalose. The addition of maltose or starch has led to a greater induction of enzyme activity than with trehalose (Table 3). However, the presence of glucose in the medium almost completely repressed the bacteria growth and synthesis of maltose-converting enzyme (data not shown).

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TABLE 3.
EFFECT OF SOME CARBOHYDRATES ADDED TO THE GROWTH MEDIUM ON ACTIVITY OF TREHALOSE PRODUCING ENZYME FROM *THERMUS RUBER*

Carbohydrate:	Total activity (U/g of cells)	Specific activity (U/mg protein)
Maltose	2.03 ± 0.18	0.086 ± 0.002
Trehalose	1.24 ± 0.05	0.053 ± 0.004
Starch	1.65 ± 0.14	0.064 ± 0.002
Sucrose	0.79 ± 0.02	0.028 ± 0.007
None	0.60 ± 0.06	0.028 ± 0.003

The results are mean values of data from four cultivations ± standard deviations.
The carbohydrate concentration in the growth medium was 0.5%.

TABLE 4.
EFFECT OF SODIUM CHLORIDE CONCENTRATION IN THE GROWTH MEDIA OF *THERMUS RUBER* ON THE CELL YIELD AND ACTIVITY OF TREHALOSE FORMATION

NaCl concentration (%)	Wet cells (g/L medium)	Total activity (U/g cells)	Specific activity (U/mg protein)
0.0	5.9 ± 0.28	0.60 ± 0.06	0.028 ± 0.07
0.5	3.5 ± 0.22	0.60 ± 0.05	0.028 ± 0.05
1.0	2.1 ± 0.22	0.49 ± 0.02	0.018 ± 0.02
1.5	2.2 ± 0.09	0.34 ± 0.01	0.010 ± 0.01

The results are mean values of data from four cultivations ± standard deviations.

Sodium chloride influenced the yield of the wet cells obtained from culture broth as well as the enzyme productivity (Table 4). A gradual decrease of the cell yield with the increase of sodium chloride concentration was observed. Furthermore, the enzyme productivity was diminished. A lower level of specific activity suggests that reduced enzyme productivity was caused not only by decreased amount of the cells in the growth medium but also by the diminished participation of maltose-converting enzyme in cell proteins (Table 4). The effect of NaCl concentration in the growth medium of *T. ruber* on the cell yield and the enzyme productivity is similar to those determined for *Thermus thermophilus* (Zdzieblo and Synowiecki 2006).

The examined enzyme is produced intracellularly and was not detected in the culture broth after cultivation of *T. ruber*. The cell-free extract from this bacteria growing without inducers had a specific activity of 0.028 U/mg protein and it was increased to up to 0.086 U/mg in the presence of 0.5% of maltose in the culture broth. The unpurified enzyme from *T. ruber* exhibits the

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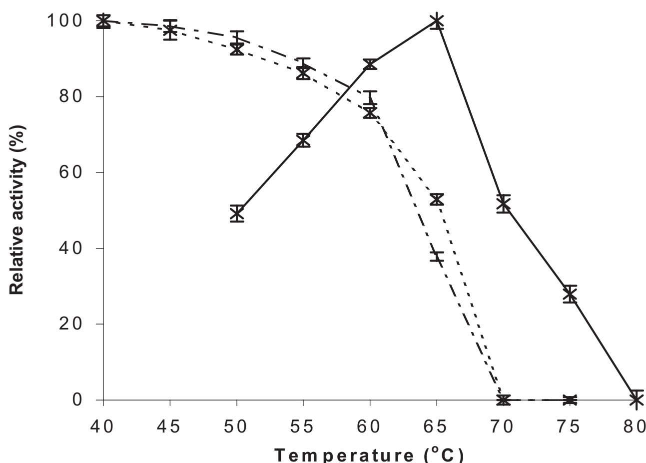

FIG. 3. EFFECT OF TEMPERATURE AT pH 6.5 ON ACTIVITY (—) AND THERMAL STABILITY AT pH 6.0 (— ·—) AND pH 7.0 (·······) OF THE ENZYME FROM *THERMUS RUBER*. THE RESULTS ARE MEAN VALUES OF THREE DETERMINATIONS

highest activity at 65C. The enzyme activity in relation to temperature has shown that 50% of the total activity was measured between 50 and 70C (Fig. 3). However, a substantial decrease of activity up to 28% of the maximal value was observed at 75C. Optimal temperature of the reaction determined for the examined enzyme is similar to that for analogous enzymes from *T. aquaticus* and *T. thermophilus* (Nishimoto *et al.* 1996a; Zdzieblo and Synowiecki 2006). To examine the heat-resistance of the enzyme from *T. ruber*, it was pre-incubated at different temperatures before determination of the activity. The enzyme activity was stable at temperatures of up to 60C, and the changes of pH from 6.0 to 7.0 almost did not influence the protein thermostabillity (Fig. 3).

The examined enzyme displayed a maximum activity at pH 6.5, similar to trehalose sythase determined by Nishimoto *et al.* (1996a). A higher pH optimum (7.5) was reported for trehalose synthase from *Pimelobacter* sp. R48 (Nishimoto *et al.* 1996b). The enzyme from *T. ruber* retained about 80% of maximal activity at pH range 6.0–7.0 (Fig. 4). Figure 5 shows the course of maltose conversion determined at increased substrate concentrations. The highest conversion of about 90% was attained at 10% maltose concentration. This value is quite similar to that (86%) reported for trehalose synthase from *Thermus caldophilus* (Koh *et al.* 1998). The high conversion of maltose indicates that the equilibrium of the reaction is favorable toward synthesis of trehalose.

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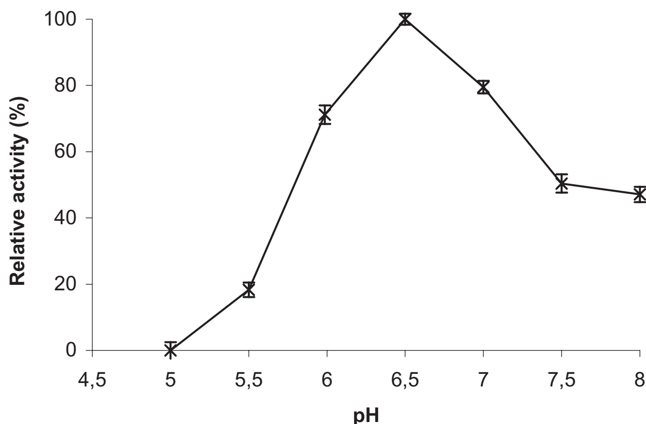

FIG. 4. EFFECT OF pH AT 65C ON ACTIVITY OF TREHALOSE-PRODUCING ENZYME FROM *THERMUS RUBER*. THE RESULTS ARE MEAN VALUES OF THREE DETERMINATIONS

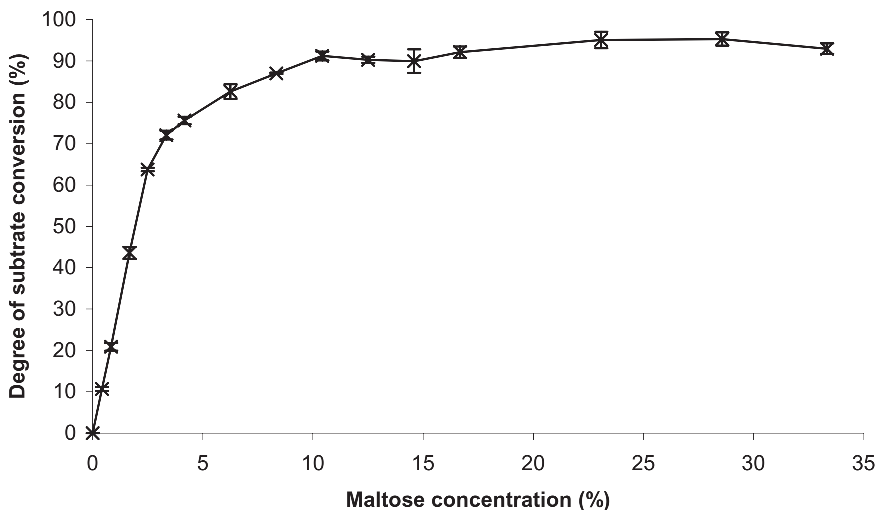

FIG. 5. EFFECT OF SUBSTRATE CONCENTRATION (%) ON MALTOSE CONVERSION CATALYZED BY TREHALOSE-PRODUCING ENZYME FROM *THERMUS RUBER* IN 0.1 M PHOSPHATE CITRATE BUFFER (pH 6.5) DURING 2 h AT 65C. RESULTS ARE MEAN VALUES OF THREE DETERMINATIONS

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CONCLUSIONS

Presented results seem to be the first study to determine the productivity and some properties of the enzyme from thermophilic bacteria, *T. ruber*, catalyzing conversion of maltose into trehalose. Our results suggest that this enzyme is a trehalose synthase. Observed thermal stability of the enzyme from these bacteria allows us to achieve a high level of substrate conversion. Furthermore, increased temperature of the process minimizes undesired microbial contamination of immobilized enzyme system. Considering substrate availability and process simplicity, the trehalose synthases is more suitable for trehalose production than enzyme systems derived from *Sulfolobus shibatae* and some other microorganisms containing MTSase and MTHase. The purification and more detailed characterization of the enzyme from *T. ruber* are currently in progress.

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