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# Enzymatic production of $\beta$ -D-glucose-1-phosphate from trehalose

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## Key words

*Beta-D-glucose-1-phosphate, bioconversion, trehalose phosphorylase, phosphorolysis, downstream processing*

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## List of abbreviations

$\beta$ Glc1P ( $\beta$ -D-glucose-1-phosphate), Tre (trehalose), Glc (glucose),  $\alpha$ Glc1P ( $\alpha$ -D-glucose-1-phosphate),  $\alpha$ Gal1P ( $\alpha$ -D-galactose-1-phosphate),  $P_i$  (inorganic phosphate)

## Abstract

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8  $\beta$ -D-glucose-1-phosphate ( $\beta$ Glc1P) is an efficient glucosyl donor for both enzymatic and  
9 chemical glycosylation reactions but is currently very costly and not available in large amounts.  
10 This paper provides an efficient production method of  $\beta$ Glc1P from trehalose and phosphate  
11 using the thermostable trehalose phosphorylase from *Thermoanaerobacter brockii*. At the  
12 process temperature of 60 °C, *Escherichia coli* expression host cells are lysed and cell treatment  
13 prior to the reaction is therefore not required. In that way, the theoretical maximum yield of 26%  
14 could be easily achieved. Two different purification strategies have been compared, *i.e.* either  
15 anion exchange chromatography or carbohydrate removal by treatment with trehalase and yeast  
16 followed by chemical phosphate precipitation. In a next step,  $\beta$ Glc1P was precipitated with  
17 ethanol but this did not induce crystallization, in contrast to what is observed with other  
18 glycosylphosphates. After conversion of the product to its cyclohexylammonium salt, however,  
19 crystals could be readily obtained. Although both purification methods were quantitative (>99%  
20 recovery), a large amount of product (50%) was lost during crystallization. Nevertheless, a  
21 production process for crystalline  $\beta$ Glc1P is now available from the cheap substrates trehalose  
22 and inorganic phosphate.  
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# 1. Introduction

Trehalose phosphorylase (EC 2.4.1.64) catalyzes the reversible phosphorolysis of trehalose (Tre) to  $\beta$ -D-glucose-1-phosphate ( $\beta$ Glc1P) and glucose (Glc) (Fig. 1). It belongs to glycoside hydrolase family 65 together with maltose phosphorylase (EC 2.4.1.8) and kojibiose phosphorylase (EC 2.4.1.230) [1]. These enzymes can be used for the production of  $\beta$ Glc1P from cheap substrates such as maltose or trehalose. Trehalose phosphorylase can be applied for the synthesis of novel disaccharides thanks to its broad acceptor specificity [2, 3]. In these reactions,  $\beta$ Glc1P functions as an activated donor for the  $\alpha$ -glucosylation of different monosaccharides. Like other glycosylphosphates, it can also be used as donor for chemical glycosylation reactions [4]. Furthermore, these compounds can have direct applications in anti-inflammatory or immunosuppressive drugs [5] or can be used to manufacture agents with vitamin D-like activity for preventing or curing skin diseases, parathyroid diseases, malignant tumors and bone diseases [6].

These potential applications have drawn considerable interest in the synthesis of glycosylphosphates. Unfortunately, their chemical synthesis suffers from a low yield and lack of anomeric selectivity [7, 8]. In that respect, biocatalytic production processes would be more advantageous. The development of galactokinase variants with modified specificity has been reported but these enzymes require ATP as phosphate donor, which hampers their large-scale application [9]. Glycoside phosphorylases, in contrast, only need inorganic phosphate to produce a glycosylphosphate. In literature, the synthesis of  $\alpha$ -glucose-1-phosphate ( $\alpha$ Glc1P) from starch [10, 11] or sucrose [12] and that of  $\alpha$ -galactose-1-phosphate ( $\alpha$ Gal1P) from lactose [13, 14] have previously been reported. A production process for  $\beta$ Glc1P has not yet been described in detail, but is similarly feasible using GH65 disaccharide phosphorylases from cheap substrates

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3 such as trehalose and maltose. Watanabe *et al.* [15] briefly mention the production of  $\beta$ Glc1P  
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5 from trehalose, using typical reaction conditions employed for  $\alpha$ Glc1P [16]. In our hands,  
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7 however, a crystalline product could not be obtained using these procedures. Therefore, we  
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9 decided to optimize the production, purification and crystallization of this compound.  
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13 The use of thermostable enzymes is desired for industrial carbohydrate conversions, as high  
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15 temperatures diminish the odds of contamination. The trehalose phosphorylase of  
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17 *Thermoanaerobacter brockii* is the most stable disaccharide phosphorylase from family GH65  
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19 that is currently available. No maltose phosphorylases have been described that can withstand  
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21 typical process conditions of 60°C for multiple hours. Also, trehalose has become a bulk product  
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23 since the development of an efficient production process from starch by Hayashibara [17],  
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25 making substrate price no longer an issue in the choice of the biocatalyst for  $\beta$ Glc1P production.  
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31 The use of a thermostable enzyme also has its benefits for the recovery of a recombinantly  
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33 expressed protein prior to the bioconversion. Indeed, permeabilization and lysis of *E. coli* cells  
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35 can be achieved upon exposure to increased temperatures, which enables the use of a whole  
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37 cell bioconversion [18]. Furthermore, endogenous *E. coli* enzymes are typically inactivated under  
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39 these conditions, which considerably reduces contaminating activities. The production of  $\beta$ Glc1P  
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41 by phosphorolysis of trehalose using both whole *E. coli* cells and cell extracts are compared in  
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43 this paper, as well as various purification methods.  
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## 2. Materials and methods

### Bacterial strains, plasmids and growth conditions

*Escherichia coli* BL21(DE3) and XL10 Gold were obtained from Stratagene. *E. coli* CGSC8974 was obtained from the Coli Genetic Stock Center (New Haven, USA). This strain is acid glucose-1-phosphatase (*agp*) negative.

The trehalose phosphorylase (*treP*) gene from *Thermoanaerobacter brockii* (accession number AB073930) was cloned into the pET15b vector (Novagen) and recombinantly expressed in *E. coli* BL21(DE3). The periplasmic trehalase gene (*treA*) from *E. coli* MG1655 (accession number ECK1185) was cloned into the pTrc99a vector (Amersham Pharmacia Biotech) and recombinantly expressed in *E. coli* XL10 Gold and *E. coli* CGSC8974.

*E. coli* was routinely grown at 37°C on LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0) supplemented with 0.1 g/L ampicillin.

Trehalose was kindly provided by Cerestar (The Netherlands). All other chemical and medium components were obtained from Sigma or BD Biosciences unless stated otherwise.

### Recombinant enzyme production

A glycerol stock of *E. coli* BL21(DE3), transformed with the pET15b-TP vector, was used to inoculate a 5 mL preculture of LB medium supplemented with ampicillin. After overnight growth, 4 mL of culture was used to inoculate 200 mL LB medium supplemented with ampicillin in a 1 L Erlenmeyer. The culture was grown to OD<sub>600</sub> 0.6 and protein expression was induced by adding IPTG to a final concentration of 0.1 mM. After 6h of protein expression, the culture

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3 (OD600 ~ 4.2) was centrifuged and the cell pellet was used either fresh for the bioconversion  
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5 reaction or frozen at -20 °C.  
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8 *E. coli* trehalase was produced in *E. coli* CGSC8974 and XL10 Gold in a similar manner from a  
9  
10 glycerol stock. Cell pellets were always frozen at -20 °C and lysed before use.  
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### 13 **Production of $\beta$ Glc1P**

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17 All bioconversion reactions were performed at 60 °C, with a substrate solution containing 200  
18 mM trehalose in 200 mM phosphate buffer pH 7. The effect of a pretreatment of the cells prior to  
19 the bioconversion was tested by performing the reaction in parallel with fresh cells, frozen cells  
20 and cell extract. These three cell pellets were obtained by centrifuging 25 mL of culture. One of  
21 these pellets was lysed by freezing it overnight and subsequently dissolving it in an enzymatic  
22 lysis solution (50 mM Tris-HCl, 1 mM EDTA, 4 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mg/mL lysozyme).  
23 After 30 min incubation at room temperature, cell debris was removed by centrifugation. To the  
24 supernatant, one frozen pellet and one fresh pellet, 50 mL of substrate was added, which was  
25 preheated at 60 °C. Bioconversion reactions were performed in 50 mL Falcon tubes using an  
26 Eppendorf Thermomixer Comfort (Eppendorf AG, Germany) with 750 rpm shaking.  
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39 To determine the  $K_{eq}$  of the phosphorolysis reaction of trehalose, a frozen cell pellet was lysed  
40 with the enzymatic lysis solution. The recombinantly produced trehalose phosphorylase  
41 possessed an N-terminal His-tag, due to the cloning in the pET15b plasmid, and was purified  
42 using Ni-NTA Spin Columns (Qiagen) with the standard native purification protocol. The purified  
43 enzyme was washed, and its buffer was changed to 30 mM MOPS pH 7 using a Microcon YM-  
44 30 Centrifugal Filter (Millipore). Bioconversion reactions were performed using substrate  
45 solutions containing 5 mM and 10 mM of  $\beta$ Glc1P and glucose in 30 mM MOPS buffer pH 7, in  
46 1.7 mL Eppendorf tubes in a heating block set at 60 °C. Both reactions were performed in  
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3 triplicate. At regular time-intervals, samples were inactivated for 10 min at 95°C in a heating  
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5 block and subsequently analyzed by colorimetrically measuring the phosphate concentration  
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7 with the phosphomolybdate method [19].  
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10 For a batch production of  $\beta$ Glc1P, 20 mL of an overnight culture of *E. coli* BL21(DE3) pET15b-  
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12 TP was used to inoculate 1 L of LB medium with ampicillin in a 2 L Biostat B reactor (B. Braun  
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14 Biotech Inc., Pennsylvania, USA) and fermentation was performed at 37°C, 400 rpm mixing and  
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16 0.8 vvm aeration. When OD600 reached 0.6, IPTG was added to a final concentration of 0.1  
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18 mM. After 16 h of protein expression, the culture (OD600 ~ 4.5) was centrifuged and the cell  
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20 pellets were added to a 5 L Biostat B reactor, containing 2 L of preheated bioconversion  
21  
22 substrate. After 2 h, the bioconversion reaction reached its equilibrium concentrations and  
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24 biomass was removed by filtration through a Seitz EKS filter. The resulting bioconversion  
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26 product was used to compare different purification methods for  $\beta$ Glc1P.  
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### 31 **Purification and crystallization of $\beta$ Glc1P**

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35 Two strategies for purification are compared in this paper. In the first strategy, separation of  
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37  $\beta$ Glc1P from phosphate, glucose and trehalose was achieved by anion-exchange  
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39 chromatography as described by Hokse [20]. Dowex 1-X8 (Alfa Aesar, Germany) in the acetate  
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41 form in a 77 x 3 cm column and a bed volume of 250 mL was used at room temperature. Elution  
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43 was done with 250 mM potassium acetate buffer pH 4.5 and fractions corresponding to 1 column  
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45 volume (250 mL) were collected. After each cycle, the resin was regenerated with 1 M  
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47 potassium acetate. Elution fractions containing  $\beta$ Glc1P were pooled and concentrated in a rotary  
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49 evaporator at 60°C. After adjustment of the pH to > 8 with KOH,  $\beta$ Glc1P was precipitated by the  
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51 addition of ethanol to form a transparent gel after overnight incubation at 4°C. This gel was  
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3 resuspended in distilled water and concentrated in a rotary evaporator to remove traces of  
4 ethanol. The resulting solution contained the pure potassium salt of  $\beta$ Glc1P.  
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9 In the second strategy,  $\beta$ Glc1P was purified by simultaneous removal of trehalose by *E. coli*  
10 trehalase (200 mL cell extract / L substrate) and glucose by baker's yeast (20 g/L) at 37°C  
11 during 24 h. Phosphate was precipitated by the addition of magnesium acetate and ammonia  
12 [16]. Biomass and precipitated phosphate were removed by filtration through a Seitz EKS filter,  
13 and  $\beta$ Glc1P was further purified by ethanol precipitation as described earlier.  
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20 To obtain a crystalline product, the cyclohexylammonium salt was prepared, as it is known that  
21 sugar-phosphates readily crystallize in this form [21-23]. The potassium cations were removed  
22 by adding Amberlite IR120H resin (Aldrich) to pH 2.5. The resin was recovered by filtration and  
23 the flow-through was titrated to pH 8 with cyclohexylamine. The cyclohexylammonium salt was  
24 crystallized by the method of Friedkin [23].  $\beta$ Glc1P crystals were filtered and dried overnight at  
25 70°C.  
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34 The hydrolysis of Glc1P by *E. coli* phosphatases present in the cell extract during carbohydrate  
35 removal by trehalase was quantified. A phosphatase positive (XL10 Gold) and a phosphatase  
36 negative *E. coli* strain (CGSC8974) were grown in both normal LB medium and LB medium  
37 supplemented with 30 mM  $\text{KH}_2\text{PO}_4$  and 2 g/L glucose. In analogy to the production of  
38 recombinant trehalase, an overnight culture was used to inoculate fresh medium. The fresh  
39 culture was grown for 6 h after an OD600 of 0.6 was reached and centrifuged. The four cell  
40 pellets thus obtained were frozen and lysed by the enzymatic lysis solution. Their phosphatase  
41 activity on both 30 mM  $\alpha$ Glc1P and  $\beta$ Glc1P in 30 mM MOPS buffer pH 7 was tested at 37°C in  
42 1.7 mL Eppendorf tubes in a heating block. Samples were inactivated for 10 min at 95°C and  
43 subsequently analyzed for their phosphate concentration using the phosphomolybdate method  
44 [19]. All reactions were performed in triplicate.  
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## Analysis of $\beta$ Glc1P

All bioconversion reactions were monitored by HPLC analysis. Samples were centrifuged to remove biomass, diluted if necessary in MilliQ water and subsequently filtrated through a Minisart RC-15 filter (pore size 0.45  $\mu$ m, Sartorius). The analysis was performed on a Prostar system (Varian) composed of a Prostar 230 Solvent Delivery Module, a cooled (4°C) Prostar 410 Autosampler, an LaChrom RI detector L-7490 (Merck) and Varian Star 6 Chromatography Workstation software. Chromatographic separation was performed with an Aminex HPx-87H Ion Exchange Column (300 x 7.8 mm, Bio-Rad) at 30°C. The eluent was 5 mM H<sub>2</sub>SO<sub>4</sub> and the flow-rate 0.6 mL/min. The expected retention times were 6.3 min ( $\beta$ Glc1P), 7.3 min (trehalose), 8.1 min (phosphate) and 8.7 min (glucose).

## 3. Results and discussion

### Production of the biocatalysts

The trehalose phosphorylase from *Thermoanaerobacter brockii* was recombinantly expressed in *E. coli*. Expression host cells can be disrupted completely to release recombinant enzymes and perform reactions of interest with cell-free extracts. These methods range from mechanical such as ultrasonication [24] to enzymatic through the use of lysozyme [25] to chemical methods by using detergents or EDTA and urea [26]. A significant advantage of the use of thermostable enzymes is the possibility to use the exposure to higher temperatures to achieve a similar effect. Thermolysis of *E. coli* to release a recombinant thermostable esterase was previously described by Ren *et al.* [18]. The host cells are lysed, and *E. coli* proteins are denatured while the thermostable enzyme remains active. This makes it possible to perform bioconversion reactions without tedious pretreatment of the cells and without significant background activities from

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3 expression host enzymes. For the biocatalytic production of  $\beta$ Glc1P specifically, this means that  
4 since the reaction is performed at 60°C, there is no product degradation by *E. coli*  
5 phosphatases. Experiments confirmed that the maximal yield of  $\beta$ Glc1P was not influenced by  
6 phosphatase activity during a bioconversion process at these temperatures.  
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12 In a second purification strategy, however, a cell extract containing recombinant trehalase is  
13 used to hydrolyse unreacted trehalose. Since the optimum temperature of this enzyme is only  
14 37°C, phosphatases remain active which could result in significant product loss. In this case, *E.*  
15 *coli* expression host phosphatase activity had to be avoided in another way. It has been  
16 described in literature that *E. coli* phosphatase activity could be lowered significantly by using an  
17 *agp*-negative strain like CGSC8974 and growing it in LB medium, supplemented with glucose  
18 and phosphate [13, 27, 28]. This effect was quantified and the specificity of *E. coli* phosphatases  
19 for  $\alpha$ Glc1P and  $\beta$ Glc1P was evaluated (Fig. 2). A large difference is visible between the  
20 hydrolysis of  $\alpha$ Glc1P and  $\beta$ Glc1P by the phosphatase positive strain. This is most significant  
21 when grown on standard LB medium, where the phosphatase activity on  $\beta$ Glc1P is 24 times  
22 lower. Both the use of a phosphatase negative strain and the addition of glucose and phosphate  
23 to the growth medium are successful in lowering Glc1P breakdown. In total, phosphatase activity  
24 on  $\beta$ Glc1P can be lowered 7 times to 0.29  $\mu$ M/min. In a 24 h purification, this will lead to  
25 hydrolysis of 418  $\mu$ M  $\beta$ Glc1P. In a mixture containing 52 mM starting concentration, this  
26 corresponds to a loss of less than 1%.  
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## 47 **Production of $\beta$ Glc1P**

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50 To determine the maximum yield of conversion, the  $K_{eq}$  of the reaction was determined  
51 experimentally by performing a bioconversion at 60°C with low substrate concentrations (5 – 10  
52 mM  $\beta$ Glc1P and glucose) in triplicate and monitoring the phosphate concentration until the  
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3 reaction stopped. The concentrations of trehalose and phosphate produced were presumed to  
4 be equimolar. Indeed, inverting phosphorylases such as TP do not display detectable hydrolytic  
5 activity, in contrast to retaining enzymes [29]. The equilibrium constant was determined by  
6 calculating  $[Glc].[\beta\text{Glc1P}]/[\text{Tre}].[P_i]$  and was equal to 0.122. This reflects the high stability of  
7 trehalose. The equilibrium constant ( $K_{eq}$ ) of the phosphorolysis reaction of glucosides, including  
8 di-, oligo- and polysaccharides is usually in the range of 0.2 or greater [12]. This implies that the  
9 reaction equilibrium of trehalose phosphorolysis lies more towards disaccharide synthesis than  
10 for other phosphorolytic reactions. Thus, using maltose could result in a higher yield of  $\beta\text{Glc1P}$ ,  
11 but then production could not be performed at the desired reaction temperature of 60°C since no  
12 stable maltose phosphorylase is available. Overall, 26% conversion of trehalose and phosphate  
13 to  $\beta\text{Glc1P}$  and glucose can be achieved. The inefficient use of trehalose is not a problem, since  
14 it has recently become a bulk product. The relative yield of  $\beta\text{Glc1P}$  compared to trehalose could  
15 be increased by using higher phosphate concentrations but that would require the removal of a  
16 much higher amount of phosphate in the downstream processing.

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35 Given that heat treatment is sufficient to permeabilize and/or lyse *E. coli* cells, it should be  
36 possible to perform the bioconversion reaction at 60°C without preliminary cell lysis. This was  
37 tested by comparing the efficiency of three bioconversion conditions using respectively fresh  
38 cells, frozen cells and cell extract (Fig. 3). From these results, it is clear that each condition  
39 possessed a different lag-phase at the start of the reaction. The reaction with cell extract started  
40 immediately and already contained 7.9 mM  $\beta\text{Glc1P}$  by the time the first sample was taken (1  
41 min), while it took the frozen cells approximately 8 min and the fresh cells 16 min to reach the  
42 same concentration. This shows that freezing the cells made them more sensitive to thermolysis  
43 and fresh cells were lysed more gradually. Although the initial velocities were different, all three  
44 reactions took equally long to reach the equilibrium concentration of 52 mM  $\beta\text{Glc1P}$  from 200  
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3 mM trehalose and phosphate, so cell lysis or freezing seems unnecessary prior to the  
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5 bioconversion.  
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8 A batch bioconversion was then performed on a 2 L scale to optimize the purification of  $\beta$ Glc1P.  
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10 The reaction was performed at 60°C with 200 mM trehalose in 200 mM phosphate buffer pH 7.  
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12 As biocatalyst, cells from a 1 L fermentation were used, which had an activity of approximately 1  
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14 U/mL of culture. After 2 h, the conversion reached its equilibrium and the cells were removed by  
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16 filtration.  
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## 19 20 21 **Purification of $\beta$ Glc1P**

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24 Two different purification methods were compared in terms of ease of use and recovery of  
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26 product. The first method was based on anion exchange chromatography as described by  
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28 Hokse [20] for the purification of  $\alpha$ Glc1P. Negatively charged phosphate and sugar-phosphates  
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30 interact with the strongly basic groups on the polystyrene-divinylbenzene matrix, while  
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32 uncharged mono- and disaccharides are not retained on the column. A typical elution profile is  
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34 presented in Fig. 4. One column volume (250 mL) of bioconversion product, containing 52 mM of  
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36  $\beta$ Glc1P and glucose and 148 mM of trehalose and phosphate, was loaded on the resin and  
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38 eluted with 250 mM potassium acetate pH 4.5. The fractions that contained  $\beta$ Glc1P were pooled  
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40 and analyzed by HPLC. This showed that all  $\beta$ Glc1P was recovered and no phosphate, glucose  
41  
42 or trehalose was present. After each cycle, the resin was regenerated with 1 M potassium  
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44 acetate. In total 1 L of bioconversion product was purified. The  $\beta$ Glc1P was precipitated by  
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46 adding ethanol to a final concentration of 80% to remove the acetate. Prior to this precipitation,  
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48 the solution had been concentrated in a rotary evaporator to minimize the loss of  $\beta$ Glc1P and  
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50 limit the amount of ethanol needed. It was found that the potassium salt of  $\beta$ Glc1P did not  
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52 crystallize, but formed a gel instead as will be discussed later. After this gel was dissolved in  
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3 distilled water and traces of ethanol were removed in a rotary evaporator, all  $\beta$ Glc1P was  
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5 recovered and highly pure.  
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9 An alternative purification method was also investigated that might be more suitable for large-  
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11 scale operations. In literature, baker's yeast *Saccharomyces cerevisiae* is often used to  
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13 metabolize sugar by-products in bioconversion reactions [30-32] while phosphate can be  
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15 removed by chemical precipitation [16]. However, Yoon *et al.* [32] have noted that trehalose is  
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17 only partially hydrolyzed by baker's yeast. This was confirmed in our experiments, which implied  
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19 the need for the addition of a trehalase to assist in carbohydrate removal. The trehalase from *E.*  
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21 *coli* was therefore overexpressed in the phosphatase negative *E. coli* strain CGSC8974 as  
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23 described earlier.  
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27 Similar to the anion exchange purification method, a 1 L batch of bioconversion product was  
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29 purified using baker's yeast and *E. coli* trehalase at 37°C. No hydrolysis of  $\beta$ Glc1P was observed  
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31 during this process, which means that contaminating phosphatase activity is negligible in both  
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33 enzyme preparations. After 24 h all trehalose and glucose were metabolized with the formation  
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35 of ethanol and some organic acids. Yeast biomass was removed by filtration. Phosphate was  
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37 precipitated from the solution in the form of struvite (magnesium ammonium phosphate) as  
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39 described by Asbhy *et al.* [16]. Struvite can be recovered from the process and has potential  
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41 applications as a slow-release fertilizer [33]. All phosphate precipitated immediately at room  
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43 temperature and was removed by filtration through a Seitz EKS filter. As previously described for  
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45 the other purification method, the  $\beta$ Glc1P solution was precipitated by adding ethanol to a final  
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47 concentration of 80% after concentrating in a rotary evaporator. HPLC analysis of the solution  
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49 showed that acetate was completely removed, but traces (< 1%) of other organic acids  
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51 remained.  
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3 Overall, both methods were successful in recovering  $\beta$ Glc1P with a high yield (> 99%). Through  
4 anion exchange, completely pure product could be obtained, while some traces of organic acids  
5 (< 1%) were still present with the alternative strategy. For scale-up of the process, the price and  
6 complexity of the procedures also needs to be considered. Although chromatographic separation  
7 is very efficient, it is a relatively sophisticated method that requires specialized equipment and  
8 careful attention. The high price of the resin is, perhaps, not problematic as it can be  
9 regenerated and re-used many times. However, a large amount of waste is generated during the  
10 elution and regeneration steps. Furthermore, the product becomes strongly diluted and the  
11 remaining phosphate and carbohydrates can not be recovered due to contamination with  
12 acetate. The main drawback of the alternative strategy is the need for trehalase as additional  
13 enzyme. Although biomass production is generally regarded as cheap, preparation of the  
14 enzyme is more labour-intensive. However, no investment in equipment is required in this case,  
15 and recovered phosphate can be valorized as highly pure struvite.  
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32 For long term storage or to remove the final impurities, crystallization of the product is desirable.  
33 Surprisingly, the potassium salt did not crystallize after addition of ethanol as mentioned earlier.  
34 This was the case for the  $\beta$ Glc1P purified by both methods and thus was not influenced by  
35 product purity. The same was true for the sodium salt, which was obtained by the addition of  
36 cation exchange resin to pH 2.5, followed by titration to pH > 8 with sodium hydroxide.  
37 Therefore, we decided to convert  $\beta$ Glc1P to its cyclohexylammonium salt by means of cation  
38 exchange, as it is known that sugar-phosphates readily crystallize in this form [21-23]. After  
39 addition of butanol and diethylether as described by Friedkin [23], small needles indeed formed  
40 in the solution. All purified product was pooled and crystallized. The formed  $\beta$ Glc1P crystals  
41 were filtered and dried overnight at 70°C, after which 22.9 g were obtained, which corresponds  
42 to a yield of approximately 50 %. In contrast to the efficient production and purification methods,  
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3 crystallization of the product results in a significant product loss. However, HPLC analysis  
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5 showed that the remaining  $\beta$ Glc1P was still present in the solution, and could be recovered by  
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7 performing a second or third harvest.  
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## 10 11 **4. Concluding remarks** 12 13

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15 Here, an efficient biocatalytic process for the production of  $\beta$ Glc1P from trehalose is described.  
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17 At the process temperature of 60°C, *E. coli* cells expressing a thermostable trehalose  
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19 phosphorylase become permeabilized for substrate and product, allowing a whole-cell  
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21 biotransformation to be performed. In that way, a maximal conversion of 26% can be readily  
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23 achieved, which is limited only by the reversible nature of the reaction. Next, two purification  
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25 methods have been optimised that are highly efficient in product recovery (> 99%). On the one  
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27 hand, anion exchange chromatography results in a very pure product but generates a lot of  
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29 waste. On the other hand, fermentation of the remaining carbohydrates followed by chemical  
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31 precipitation of phosphate is a simpler process but results in product that is slightly less pure.  
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33 Finally, crystallization of  $\beta$ Glc1P was found to be possible only as cyclohexylammonium salt, in  
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35 contrast to what is observed for other glycosyl phosphates.  
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## 40 41 **Acknowledgements** 42 43

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50 The authors have declared no conflict of interest.  
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For Peer Review

## Figure Legends

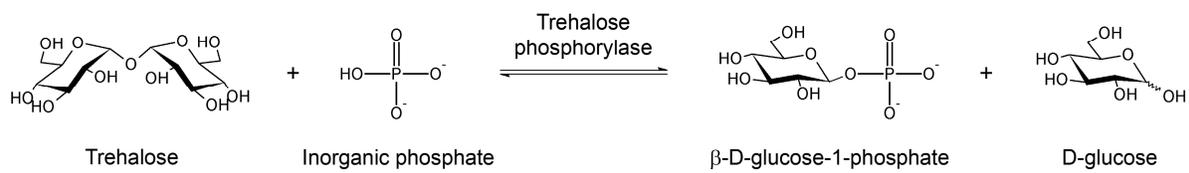
Figure 1. Reaction scheme of trehalose phosphorylase.

Figure 2. Comparison of phosphatase activity on  $\alpha$ Glc1P and  $\beta$ Glc1P in an *agp*-positive (XL10 Gold) and negative (CGSC8974) *E. coli* strain, grown on standard LB medium and LB medium supplemented with phosphate (30 mM  $\text{KH}_2\text{PO}_4$ ) and glucose (2 g/L).

Figure 3. Effect of cell pretreatment on the phosphorolysis of trehalose. Cells were centrifuged and either immediately resuspended in preheated substrate, frozen or frozen and lysed with an enzymatic lysis solution before adding preheated substrate (200 mM trehalose in 200 mM phosphate buffer pH 7).

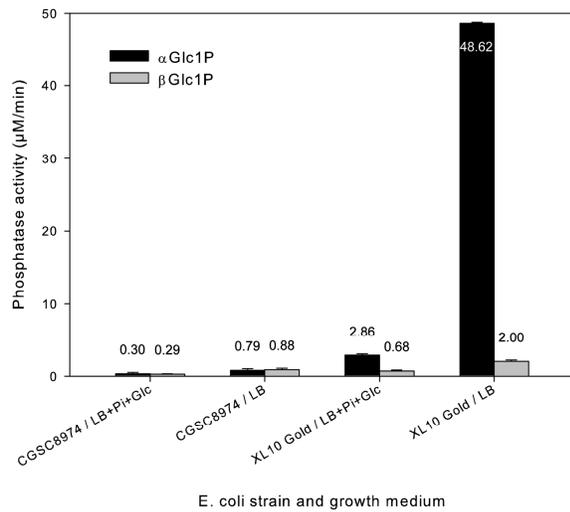
Figure 4. Anion exchange elution profile for the separation of  $\beta$ Glc1P from trehalose, glucose and phosphate. Separation was performed on Dowex 1-X8 resin with 250 mM potassium acetate pH 4.5 as eluent. Each fraction corresponds to one column volume (250 mL).

Figure 1.



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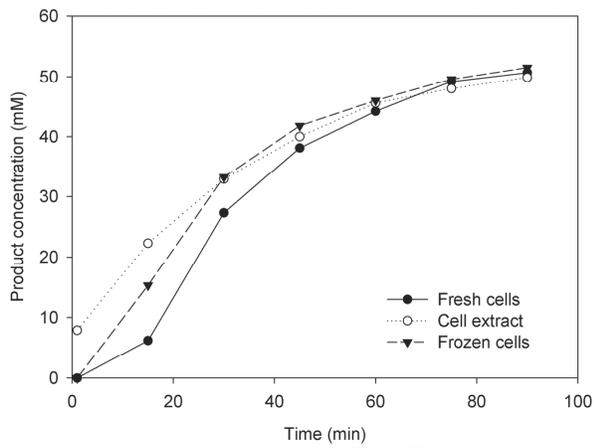
Figure 2.



E. coli strain and growth medium

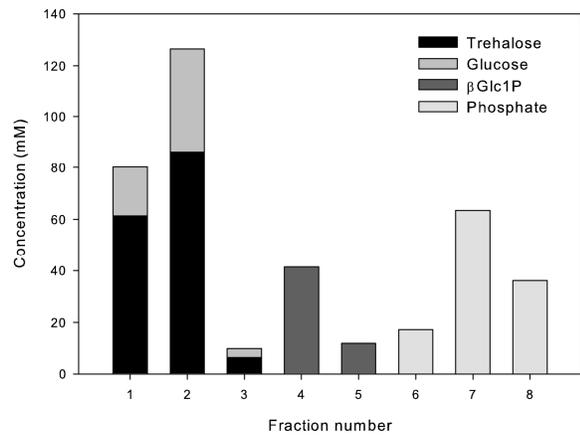
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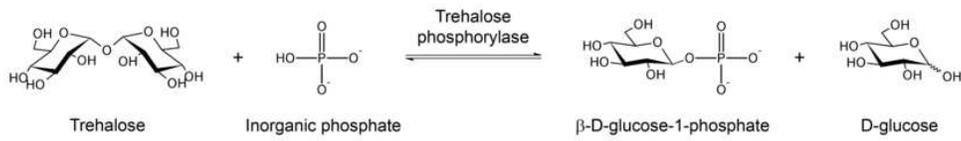
Figure 3.



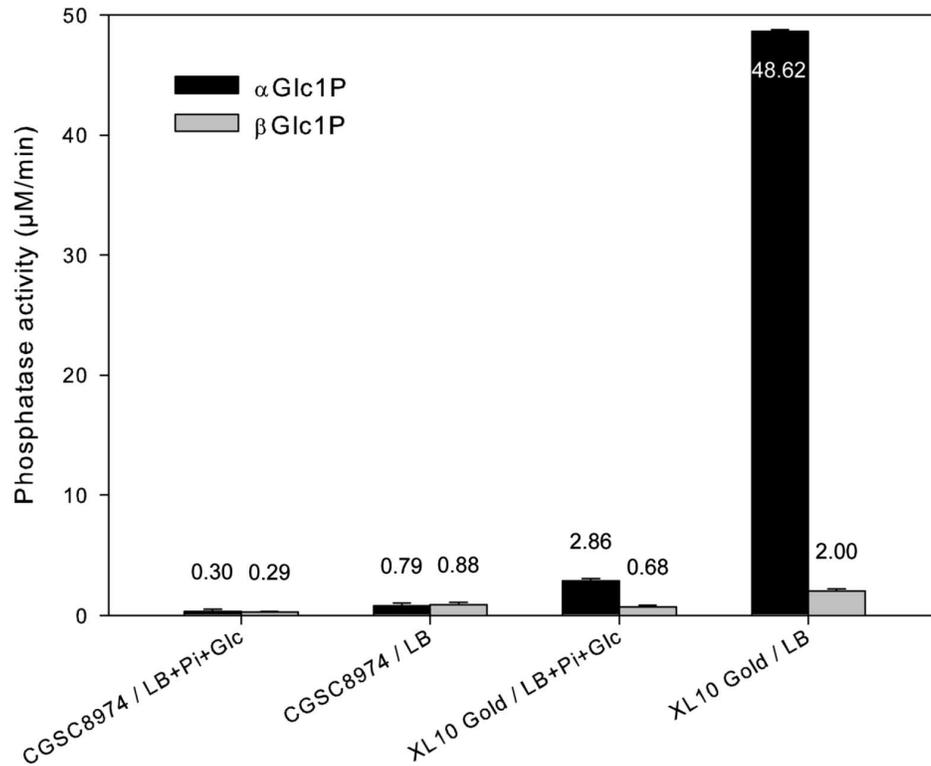
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Figure 4.



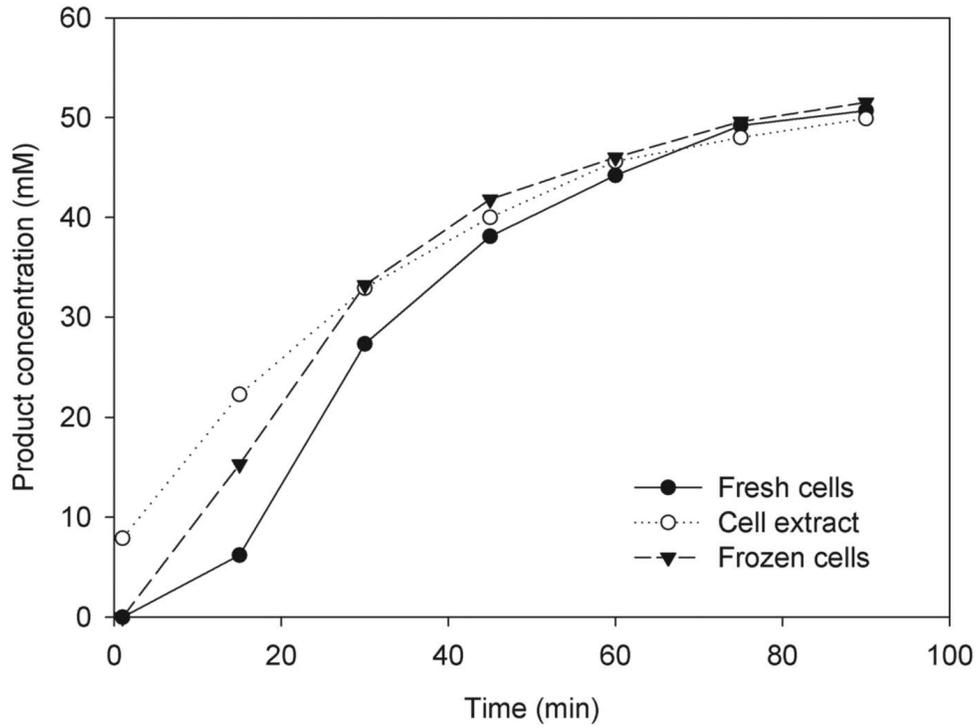


Reaction scheme of trehalose phosphorylase.  
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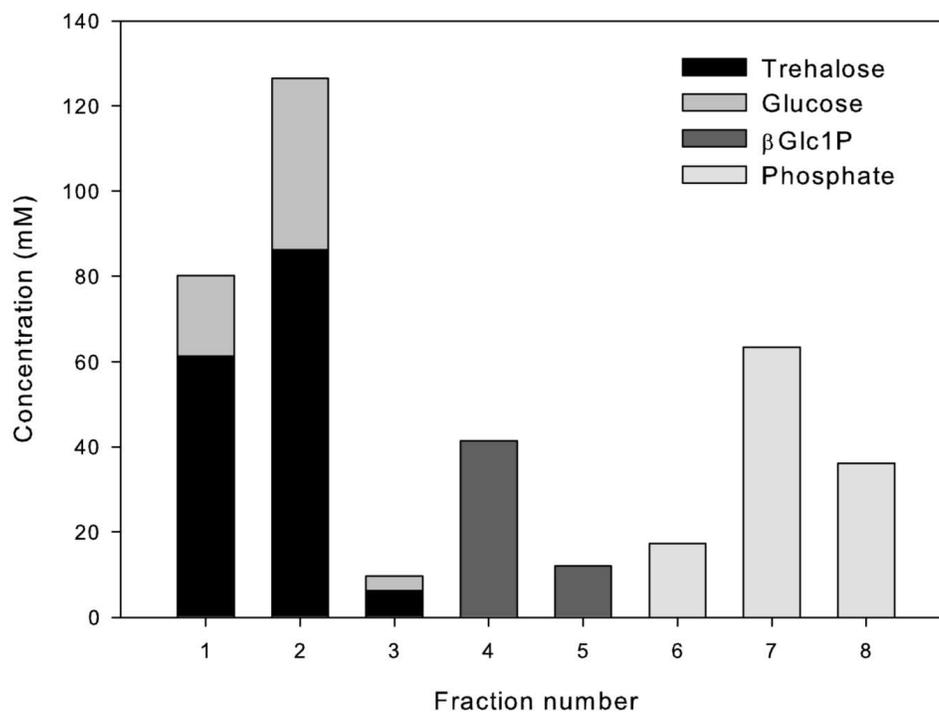


E. coli strain and growth medium

Comparison of phosphatase activity on  $\alpha$ Glc1P and  $\beta$ Glc1P in an agp-positive (XL10 Gold) and negative (CGSC8974) *E. coli* strain, grown on standard LB medium and LB medium supplemented with phosphate (30 mM  $\text{KH}_2\text{PO}_4$ ) and glucose (2 g/L).  
70x63mm (600 x 600 DPI)



Effect of cell pretreatment on the phosphorolysis of trehalose. Cells were centrifuged and either immediately resuspended in preheated substrate, frozen or frozen and lysed with an enzymatic lysis solution before adding preheated substrate (200 mM trehalose in 200 mM phosphate buffer pH 7).  
60x46mm (600 x 600 DPI)



Anion exchange elution profile for the separation of  $\beta$ Glc1P from trehalose, glucose and phosphate. Separation was performed on Dowex 1-X8 resin with 250 mM potassium acetate pH 4.5 as eluent. Each fraction corresponds to one column volume (250 mL).  
60x46mm (600 x 600 DPI)