博 士 論 文 の 要 約

博士の専攻分野の名称： 博　士（農学）　　氏名　Gao Yu

学 位 論 文 題 名

Functional analysis of maltose phosphorylase MalE from *Bacillus* sp. AHU2001 and synthesis of oligosaccharides and sugar phosphates with the enzyme

（*Bacillus* sp. AHU 2001 由来マルトースホスホリラーゼ MalE の機能ならびに本酵素を用いたオリゴ糖および糖リン酸の合成に関する研究）

Glycoside phosphorylases (GPs) are a group of carbohydrate-active enzymes that transfer a glycosyl moiety from the non-reducing end of a polysaccharides or oligosaccharides to inorganic phosphate (Pi), to generate a glycosyl phosphate. Their phosphorolytic reaction is reversible and practical for synthesis of sugars from appropriate glycosyl phosphate and an acceptor. GPs are categorized into two types in terms of anomeric configuration of the glycosyl donor and the resulting glycosyl phosphate. Retaining GPs do not change the anomeric configuration between the substrate and the sugar phosphate, while inverting phosphorylases change the anomeric configuration in the product. All GPs are classified into ten families of carbohydrate active enzymes database (CAZy). Retaining enzymes are in two glycosyltransferases (GT) families, GT35 and GT4, and two glycoside hydrolases (GH) family, GH3 and GH13. Inverting enzymes are found only in GH families: GH65, GH94, GH112, GH130, GH149, and GH161. Inverting GPs cleave the glucosidic linkage via the single displacement mechanism, in which the general acid catalyst donates a proton to the scissile glucosidic oxygen, and the Pi attacks the anomeric carbon of the Glc residue in subsite -1.

GH65 is a family of inverting phosphorylases acting on α-glucosides to produce β-D-glucose 1-phosphate (β-Glc1*P*). With exception of isomaltose (Isomal), all other α-linked glucobioses are phosphorolytically processed by the corresponding inverting GPs in GH65. GH65 contains maltose phosphorylase (MP, EC 2.4.1.8), trehalose phosphorylase (TP, EC 2.4.1.64), trehalose 6-phosphate phosphorylase (T6PP, EC 2.4.1.216), kojibiose phosphorylase (KP, EC 2.4.1.230), nigerose phosphorylase (NP, EC 2.4.1.279), 1,3-α-glucosyl-L-rhamnose phosphorylase (EC 2.4.1.282), 1,2-α-glucosylglycerol phosphorylase (GGP, EC 2.4.1.332), and

1,3-α-oligoglucan phosphorylase (EC 2.4.1.334), as well as acid trehalose hydrolase (EC 3.2.1.28) from eukaryotes. Structures of GH65 enzymes, *L. brevis* MP, *C. saccharolyticus* KP, *B. selenitireducens* GGP, and *Thermoanaerobacter* sp. TP, have been solved thus far. The GH65 enzymes have an $(\alpha/\alpha)_6$-barrel catalytic domain similar to those of GH15 inverting glycosidases, including glucoamylase (EC 3.2.1.3) and glucodextranase (EC 3.2.1.70). They form the clan GH-L. In GH65 phosphorylases, Pi is located in the active site at the position, corresponding to the general base catalyst Glu residue of GH15 glycosidases. The Glu residue on the loop connecting the fifth and sixth α-helices of the catalytic domain serves as the general acid catalyst and conserved in the enzymes of GH65 and GH15.

MP catalyzes the reversible phosphorolysis of maltose (Mal) to β-Glc1*P* and D-glucose (Glc). MP has been found in many bacteria. These bacteria utilize MP for the intracellular metabolism of Mal/maltodextrin. *Bacillus subtilis* and *Lactobacillus acidophilus* utilize MP along with α-glucosidase (EC 3.2.1.20) and neopullulanase (EC 3.2.1.135) for the intracellular metabolism of maltodextrin. The catalytic general acid residue of LbMP is Glu487, which is situated at the center of the active pocket. Although the structure of MP in complex with Mal is not available, structural comparison between *L. acidophilus* MP and *Thermoanaerobacterium thermosaccharolyticum* glucoamylase suggested that His413 and Glu415 on loop 3, that connects the third and fourth α-helices of the $(\alpha/\alpha)_6$–catalytic domain, are involved in the binding to the reducing end Glc residue of Mal in subsite +1.

Several non-naturally occurring α-(1→4)-glucosides have been synthesized by reverse phosphorolysis of MPs. Some α-(1→4)-glucosides enhanced the growth of *Bifidobacteria animalis* subsp. lactis and *Bifidobacterium longum*. It has also been reported that β-Glc fluoride can be an alternative of β-Glc1*P* in the reverse phosphorolysis. *Lactobacillus brevis* MP transferred Glc residue from β-Glc fluoride to α-Glc to produce α-Mal and hydrogen fluoride. Several MPs show relatively high activity of reverse phosphorolysis to acceptor of Glc derivatives at the 2-C and 6-C positions, including D-glucosamine (GlcN), *N*-acetyl-D-glucosamine (GlcNAc), and D-xylose (Xyl), and form α-(1→4)-glucosides. MP can synthesize β-Glc1*P* from Mal, which can be easily produced from abundant starch. By coupling the reactions of MP and other α-GPs, several oligosaccharides are produced from Mal. Trehalose (Tre) and nigerose (Nig) were produced by the coupling reactions of MP and TP, and MP and NP, respectively.

The gene *malE* that encodes a GH65 enzyme is found in a neighboring gene of the GH31 α-glucosidase gene in *Bacillus* sp. AHU2001 (formerly known as *Bacillus* sp. SW20). The gene is downstream of five open reading frames *malA*, *malB*, *BspAG31A*, *malC*, and *malD*. MalA and MalB encode the proteins involved in

ATP-binding cassette (ABC)-type sugar transport system. Thus, these proteins are predicted to be involved in the uptake of maltooligosaccharides. MalC and MalD are predicted to be transcriptional repressor. The deduced amino acid sequence of MalE share 52–60% identity with that of characterized MPs: *B. selenitireducens* MP (GenBank number, ADH99560.1), *Bacillus* sp. RK-1 MP (GenBank number, BAC54904.1), *Enterococcus faecalis* MP (GenBank number, AAO80764.1), *L. acidophilus* MP (GenBank number, AAV43670.1), *L. brevis* MP (Uniprot number, Q7SIE1), *Lactobacillus sanfranciscensis* MP (GenBank number, ADH99560.1), and *Paenibacillus* sp. SH-55 MP (GenBank number, BAD97810.1). It is speculated that although short maltooligosaccharides can be hydrolyzed completely to Glc by BspAG31A solely, the presence of MalE indicates that a part of Mal, resulting from the hydrolysis of maltooligosaccharides catalyzed by BspAG31A, is metabolized thorough phosphorolysis. In this work, the malE-gene product, putative GH65 MP, was characterized to address the predicted involvement in maltooligosaccharides metabolism in *Bacillus* sp. AHU2001. Through the detailed acceptor specificity analysis, the hidden ability of this GH65 enzyme was classified, together with the possible binding mode on this protein structure. The ability was applied for the enzymatic synthesis of oligosaccharides including novel compounds and sugar phosphates using MalE.

The recombinant MalE protein was successfully produced in *E. coli* BL21 (DE3) transformant. From *E. coli* cells proliferated in 2 L of culture broth, cell-free extract was prepared by sonication. The cell-free extract (34 mL, 2660 mg of protein) included 717 U of activity (specific activity was 0.27 U/mg). Recombinant MalE was purified by Ni^{2+}-affinity column chromatography. The yield of the purified enzyme was 13.6 mg. The specific activity of purified enzyme to 4 mM maltose and 10 mM sodium phosphate buffer (pH 8.0) at 37°C was 15.9 U/mg. The molecular mass of MalE was 90 kDa as determined by SDS-PAGE (theoretical molecular mass is 89.6 kDa), and 193 kDa as determined by gel-filtration column chromatography. This indicated that MalE exists as homodimer under nondenaturing conditions similar to other MPs. Unlike the reaction to Mal, phosphorolytic activity of MalE to Tre, Koj, Nig, Isomal, and maltotriose was not detectable (less than 0.089 μmol/min/mg, which is 0.56% of that to Mal), indicating that MalE is specific to the α-(1→4)-glucobiose similar to other MPs. MalE exhibited the highest phosphorolytic activity at pH 8.1 on 4 mM Mal and 10 mM Pi. The optimal pH of MalE is higher than many MPs from other origins, which showed optimal pH at ≤7.0. The enzyme showed the highest activity at 45°C. The residual activity was higher than 80% of the original activity in a pH range of 4.5–10.4 after the pH treatment at 4°C for 24 h and at ≤40°C after the heat treatment at pH 8.0 for 15 min.

In the phosphorolysis of Mal, the curves obtained by plotting 1/[Pi] versus 1/v at various Mal concentrations were linear and intersected at a point on the second quadrant. This result indicates that MalE catalyzed the phosphorolytic reaction of Mal through a sequential bi bi mechanism, involving the formation of a ternary complex as the reactions of other inverting disaccharide phosphorylases. The Glc donor is the second substrate in the direction of phospholysis of Mal. The calculated kinetic parameters were as follows: k_{cat}, 30.9 ± 0.6 s^{-1}; K_{mA}, 0.295 ± 0.059 mM; K_{mB}, 0.835 ± 0.123 mM; and K_{iA}, 9.07 ± 1.74 mM (A, Pi; B, Mal).

Acceptor specificity of the reverse phosphorolysis was determined by measuring initial reaction rates to 10 mM β-Glc1*P* and 10 mM various acceptors. Compared to the reaction without acceptor, which β-Glc1*P* was the only substrate, the production velocity of Pi was increased by the addition of Glc, Man, All, 2-deoxyGlc, 3-deoxyGlc, 6-deoxyGlc, Fuc, Sor, Lyx, Xyl, Koj, α-MG, GlcN, GlcNAc, and 1,5-AG as acceptors. Such enhancement was not observed in the reactions with Gal, Tal, Alt, Ara, 4-deoxyGlc, Rha, Fru, Tag, and Xul, Tre, Nig, Mal, Isomal, β-MG, and GlcA.

The apparent kinetic parameters for the reverse phosphorolysis with Glc, Man, All, 2-deoxyGlc, 3-deoxyGlc, 6-deoxyGlc, Fuc, Sor, Lyx, Xyl, Koj, α-MG, GlcN, GlcNAc, and 1,5-AG were determined based on the Pi-releasing velocity in the presence of 10 mM β-Glc1*P*. In the reverse phosphorolysis with Glc, Man, 2-deoxyGlc, 3-deoxyGlc, 6-deoxyGlc, Xyl, Koj, GlcN, and GlcNAc, the hydrolysis of β-Glc1*P* can be neglected, and the apparent kinetic parameters for acceptor were determined by fitting the Michaelis-Menten equation to the reaction velocity at various concentrations of acceptor. On the other hand, the hydrolytic activity of β-Glc1P was not negligible in the reactions to All, Fuc, Sor, Lyx, α-MG, and 1,5-AG due to low activity of reverse phosphorolysis. Thus, the reaction equation, obtained from the reaction scheme, in which both hydrolysis of β-Glc1P and the reverse phosphorolysis occur, was employed to determine the apparent kinetic parameters. The reaction velocity for 10 mM β-Glc1*P* (v_0) was determined to be 0.088 s^{-1} from the kinetic parameters of β-Glc1*P* hydrolysis in the absence of acceptors: k_{cat1}, 0.983 ± 0.029 s^{-1}; and K_s, 28.9 ± 1.1 mM. Velocities for all the tested acceptors followed well the equation. MalE exhibited high $k_{cat(app)}/K_{m(app)}$ for Glc, GlcN, and 6-deoxyGlc (12.8, 15.2, and 12.2 $s^{-1}mM^{-1}$, respectively). GlcN was the most favorable substrate than Glc in reverse phosphorolysis due to its higher $k_{cat(app)}/K_{m(app)}$ value than that of Glc. Even at high acceptor concentration, GlcN shows higher $k_{cat(app)}$. Furthermore, 6-deoxyGlc possessed almost similar affinity to Glc. The $k_{cat(app)}/K_{m(app)}$ value for the other tested acceptors was 0.23–12% of that to Glc, because of lower $k_{cat(app)}$ and higher $K_{m(app)}$ values.

To understand the substrate binding mechanism, the binding mode of Glc in subsite +1 of MalE was predicted through structural comparison between a model structure of MalE and the crystal structure of *Caldicellulosiruptor saccharolyticus* KP. Model structure of MalE was constructed through a homology modeling with Phyre2 program. In the model structure of MalE, the orientation of the amino acid residues in the substrate binding site was consistent well with that of the template protein (*L. brevis* MP; amino acid sequence of MalE is 52% identical with that of *L. brevis* MP). The superimposition showed that the amino acid residues in subsite −1 of MalE, including the general acid catalyst Glu486, were located at similar position to the corresponding residues of *C. saccharolyticus* KP. This suggests that MalE and the KP share the binding mode of non-reducing end Glc residue, meaning that the glucosidic oxygen (4-O and 2-O of the reducing end Glc residue of Mal and Koj, respectively) is situated at the same position in these enzymes. To predict the binding mode of Mal, α-Glc, which is utilized as acceptor of MP in the reverse phosphorolysis, was superimposed onto the reducing end Glc residue of Koj in subsite +1. His415, Glu417, and Lys595 of MalE were predicted to form hydrogen bonds with 5-O, 1-O, and 3-O of the superimposed Glc in subsite +1, respectively. The hydrogen bonds formed by Glu417 and Lys595 are consistent with the prediction using *L. acidophilus* MP. In addition to these residues, Tyr351 is predicted to form a hydrogen bond with 3-O of Glc. As the residues of MalE, predicted to be involved in the formation of subsite +1, are conserved in MPs, the substrate binding mode is presumably common to MPs.

The $k_{cat(app)}/K_{m(app)}$ for reverse phosphorolysis of GlcN by MalE was as high as that of Glc (15.2 and 12.8 $s^{-1}mM^{-1}$, respectively), although the $k_{cat(app)}/K_{m(app)}$ values for 2-deoxyGlc and Man were 16- and 61-fold lower than that for Glc. This indicated that the equatorial 2-hydroxy group or amino group are required for good acceptors. MalE utilized GlcNAc harboring equatorial bulky chemical group at the 2-C position as acceptor, although its $k_{cat(app)}/K_{m(app)}$ value was as low as 2-deoxyGlc and Man. Other MPs of *Neisseria perflava*, *L. acidophilus*, and *Bacillus selenitireducens* are also reported to utilize GlcNAc as acceptor in reverse phosphorolytic reaction. Furthermore, MalE acted on Koj [α-D-Glcp-(1→2)-Glcp] as an acceptor with low $k_{cat(app)}/K_{m(app)}$. The reducing end of Glc, 2-O of which is glucosylated, was thought to serve as acceptor, as *B. selenitireducens* MP, glucosylates the reducing end Glc residue of Koj through the reverse phosphorolysis. The model structure of MalE suggests no interaction with 2-O of Glc in subsite +1, and has open space near the 2-O of this Glc. This space could account for the activity of reverse phosphorolysis to the derivatives of Glc with bulky chemical group at the 2-C position. The subsite +1 of MalE presumably has enough space to accommodate the acceptor with an equatorial

bulky chemical group at the 2-C position.

The $k_{cat(app)}/K_{m(app)}$ of MalE for 6-deoxyGlc (12.2 $s^{-1}mM^{-1}$) was comparable with that of Glc (12.8 s^{-1} mM^{-1}). However, the $k_{cat(app)}/K_{m(app)}$ of MalE for Xyl (1.57 s^{-1} mM^{-1}) was considerably lower. This suggests that 6-C, not 6-OH, of Glc is involved in binding at subsite +1. In the modeled structure of MalE, Met406 is situated near the hydroxymethylene group of Glc in subsite +1 to have a hydrophobic interaction. The requirement of 6-C for the binding of MalE is higher than *B. selenitireducens* MP, which exhibited reverse phosphorolysis activity to Xyl comparable to that to Glc. In contrast to the reaction with the Glc derivatives of 2-C and 6-C positions, MalE exhibited very low synthetic activity to all the 1- and 3-OH Glc derivatives tested: 1,5-AG, α-MG, 3-deoxyGlc, and All. In addition, the tested 4-OH Glc derivatives, 4-deoxyGlc and Gal, were not able to be utilized as acceptor. This indicates that 1-, 3- and 4-hydroxy groups of Glc are important for the subtract binding. Tyr351, Glu417, Glu486 and Lys595, predicted to interact with these hydroxy groups, are thought to be essential for the substrate binding. Judging from Glu417 forming a hydrogen bond with the 1-O hydroxyl of the α-anomer at subsite +1, MalE might act only on the α-anomeric configuration of Mal but not on β-Mal.

Oligosaccharides were prepared through the reverse phosphorolysis of MalE. In the reaction with Man, GlcN, GlcNAc, and Xyl (starting substrates: 5 mmol of β-Glc1*P* and acceptor), the obtained amount of the oligosaccharides after the purification was 1.3 mmol (26% yield), 0.66 mmol (13% yield), 2.0 mmol (39% yield), and 2.4 mmol (49% yield), respectively. The yield of oligosaccharides from 100 μmol of β-Glc1*P* and acceptors (All, Lyx, 2-deoxyGlc, and Sor) was 33 μmol (33% yield), 61 μmol (61% yield), 38 μmol (38% yield), and 76 μmol (76% yield), respectively. The chemical structure of the oligosaccharides prepared was analyzed by ESI-MS and NMR. The molecular mass of the reaction products from Man, GlcN, GlcNAc, All, Xyl, Lyx, 2-deoxyGlc, and Sor was 365 m/z $[M + Na]^+$, 380 m/z $[M + K]^+$, 406 m/z $[M + Na]^+$, 365 m/z $[M + Na]^+$, 335 m/z $[M + Na]^+$, 335 m/z $[M + Na]^+$, 349 m/z $[M + Na]^+$, and 381 m/z $[M + K]^+$, respectively, indicating that all the oligosaccharides were disaccharides. In HMBC-spectrum, correlation peak between C1 of the non-reducing end Glc residue and H4 of Man, GlcN, GlcNAc, All, Xyl, Lyx residue, and between H1 of a Glc residue and C4 of a Man, GlcN, GlcNAc, All, Xyl, Lyx residue, were observed, indicating that the glucosidic linkages was formed between C1 of the Glc and C4 of Man, GlcN, GlcNAc, All, Xyl, and Lyx. The $J_{H1, H2}$ values of Glc residue of the products from Man, GlcN, GlcNAc, All, Xyl, Lyx were 3.90, 3.85, 3.90, 3.50, 3.85, and 4.00 Hz, respectively, indicating the formation of α-linkage. Taken together, the reaction products from Man, GlcN, GlcNAc, All, Xyl, and Lyx were determined to be α-D-Glcp-(1→4)-D-Man, α-D-Glcp-(1→4)-D-GlcN,

α-D-Glcp-(1→4)-D-GlcNAc, α-D-Glcp-(1→4)-D-All, α-D-Glcp-(1→4)-D-Xyl, α-D-Glcp-(1→4)-D-Lyx, respectively. Correlation peak between C1 of the non-reducing end Glc residue and H3 of 2-deoxyGlc and Sor residue, and between H1 of the Glc residue and C3 of a 2-deoxyGlc and Sor residue, was observed in the HMBC-spectrum. The $J_{H1,\ H2}$ value of Glc residue was 3.70 and 3.85 Hz. These results indicated that MalE produced α-D-Glcp-(1→3)-2-deoxyGlc and α-D-Glcp-(1→3)-L-Sor from 2-deoxyGlc and Sor, respectively. This suggested that 2-deoxyGlc in $^{3}C_{O}$ may bind to subsite +1 upside down, and 3-OH group serves as the nucleophile during the reverse phosphorolysis. MalE exhibited weak but apparent reverse phosphorolysis activity to a ketose, Sor, and catalyzed the formation of α-(1→3)-glucoside, indicating that 3-O of Sor, acted as the nucleophile in the reverse phosphorolysis. As 1-C, 1-O, 2-C, 3-C, 3-O, 4-C, 4-O, 5-C, 5-O, 6-C and 6-O of α-L-sorbopyranose could be located at positions similar to 6-C, 6-O, 5-C, 4-C, 4-O, 3-C, 3-O, 2-C, 2-O, 1-C, and 5-O of Glc, respectively, α-L-sorbopyranose presumably served as the acceptor in this reaction.

The disaccharides prepared from 2-deoxyGlc, Man, GlcN, GlcNAc, All, Xyl, Lyx, and Sor were digested by rat intestinal α-glucosidases. The Glc-releasing velocity (digestion rate) was compared with that of Mal. The hydrolytic velocities (bond-cleaving velocities) of α-D-Glcp-(1→4)-D-Man, α-D-Glcp-(1→4)-D-GlcN, α-D-Glcp-(1→4)-D-GlcNAc, α-D-Glcp-(1→4)-D-All, α-D-Glcp-(1→4)-D-Xyl, α-D-Glcp-(1→4)-D-Lyx, α-D-Glcp-(1→3)-D-2-deoxyGlc, and α-D-Glcp-(1→3)-L-Sor were 43.8 ± 2.5%, 39.8 ± 2.0%, 40.4 ± 0.3%, 37.9 ± 0.8%, 45.0 ± 0.3%, 39.3 ± 1.2%, 28.1 ± 0.9%, and 28.0 ± 0.7% of that of Mal, respectively. The tested α-(1→3)-glucosides exhibited even higher tolerance than the others. All compounds showed higher tolerance than Mal, particularly. This indicated that these oligosaccharides could reach large intestine. It has been reported that α-D-Glcp-(1→4)-D-GlcN, α-D-Glcp-(1→4)-D-GlcNAc, α-D-Glcp-(1→4)-D-Xyl promoted the growth of *B. animalis* and *B. longum*, which were involved in enhancing gut health and prevent gastrointestinal infections in humans. The other synthesized α-(1→4)-glucosides: α-D-Glcp-(1→4)-D-Man, α-D-Glcp-(1→4)-D-All, and α-D-Glcp-(1→4)-D-Lyx, may play an important role in prebiotic oligosaccharides. As α-D-Glcp-(1→3)-D-2-deoxyGlc and α-D-Glcp-(1→3)-L-Sor exhibited higher tolerance against the α-glucosidase than the α-(1→4)-glucosides, they might also act as prebiotic oligosaccharides.

β-Glc1*P* was produced from 0.1 M Mal and 0.4 M Pi in the presence and absence of bakery yeast. In the reaction without bakery yeast, β-Glc1*P* concentration of the reaction mixture reached plateau at reaction 24 h, and the yield of β-Glc1*P* was 47% at 72 h reaction. The yield is calculated from the ratio of β-Glc1*P* concentration

to the starting Mal concentration. Addition of bakery yeast to the reaction mixture reduced the concentration of Glc, which was produced through the phosphorolysis of Mal, and enhanced the production of β-Glc1*P*. Production yield of the reaction with bakery yeast reached 62% at 72 h reaction. After 72 h, since Glc was consumed up by bakery yeast, concentration of β-Glc1P was not increased, indicating optimal time was 72 h in the presence of bakery yeast. Meanwhile, β-Glc1*P* concentration was stable at about 46 mM after 48 h without bakery yeast. Obviously, adding bakery yeast to the mixture can promote yield of β-Glc1*P*, but yield only reached to 62% was not high as expected. Mal can be fermented by bakery yeast through their inducible system in the membrane, but if the medium contained Glc and Mal, the yeast ferments Glc priory. In the reaction mixture, bakery yeast grew by utilizing produced Glc firstly. When large amount of bakery yeast grew up and consumed majority of Glc after 48 h, the yeast might start to use Mal. This may lead to the equilibrium of phosphorolysis decreased and prevented converting to β-Glc1*P* and Glc.

Glc6*P* was produced from 0.1 M Mal and 0.1 M phosphate buffer (pH 7.0) by the coupling reaction of MalE and β-PGM. The production of Glc6*P* in the presence and absence of bakery yeast reached the maximum at 48 h reaction, and the yields of Glc6*P* were 80% (with bakery yeast) and 68% (without bakery yeast). The yield is calculated from the ratio of produced Glc6*P* concentration to the starting Mal concentration. Glc6*P* concentration slightly decreased in the reaction with bakery yeast, whereas the concentration was not changed in the reaction without bakery yeast.

The equilibrium constant of reaction from β-Glc1*P* to Glc6*P* by β-PGM, calculated from [Glc6P]/[β-Glc1P], was 18.5-28.6, indicating that β-PGM can convert almost completely β-Glc1*P* into Glc6*P*. Glc6*P* could be produced efficiently from Mal by coupling reaction using MalE and β-PGM in the presence of yeast. As the Glc6*P* preparation result showed, Glc6*P* was reached to maximum 80% yield (calculated from [Glc6P]/[Mal]) at 48h. The yield is higher than that of preparing by hexokinase (EC 2.7.1.1) from Glc and by phosphoglucose isomerase (EC 5.3.1.9) from fructose 1,6-disphosphate in mild acid, which only reached to 65% and 70%, respectively. In view of yield and cost, producing Glc6*P* from Mal using MalE and β-PGM in addition of bakery yeast it is feasible.