

Article

The Synthesis of Mannose-6-Phosphate Using Polyphosphate-Dependent Mannose Kinase

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Abstract: Mannose-6-phosphate (M6P) is involved in many metabolic pathways in life, and it has important applications in the treatment of diseases. This study explored a cost-effective enzyme catalytic synthesis method of M6P, using polyphosphate-dependent mannose kinase from *Arthrobacter* species. This synthesis uses polyphosphate to replace expensive ATP, and it is greener and safer than chemical synthesis. This study investigated the effects of key factors such as metal ions, temperature, and substrate addition on this enzymatic reaction, and improved the conversion efficiency. We moreover take advantage of the response surface method to explore the best catalytic conditions synthetically. The conversion was 99.17% successful under the optimal reaction conditions. After a series of optimizations, we carried out a 200 mL scale-up experiment, which proved that the method has good prospects for industrial applications.

Keywords: mannose-6-phosphate; polyphosphate-dependent mannose kinase; polyphosphate

1. Introduction

Mannose-6-phosphate (M6P), also known as 6-phosphate mannose, is involved in many metabolic pathways in living organisms, such as gluconeogenesis, fatty acid synthesis, and amino acid synthesis [1]. Mannose-6-phosphate also plays an important role in the cell. Mannose-6-phosphate is produced during the formation of lysosomal enzymes by the transfer of phosphate groups to mannose by acetylamino-1-phosphate. It is formed on the terminal mannose residue and is an important marker substance of glycoproteins [2].

M6P has a wide range of applications in the treatment of many diseases, because mannose-6-phosphate acts on the mannose-6-phosphate receptor (M6PR) [3] which indirectly affects the cellular signaling pathway. The mannose-6-phosphate receptor binds to a variety of cell membrane surfaces as well as signaling molecules of intracellular signaling pathways. Studies have shown that the mannose-6-phosphate receptor is closely related to many metabolic pathways in cells [4].

M6P and its analogue can be synthesized through mostly chemical and enzymatic methods. Garcia et al. prepared an analog of M6P by Swern oxidation and benzyl protection reactions in which methyl 2,3,4-tri-*O*-benzyl- α -D-mannopyranoside was used as starting material, in a total of two to three reaction steps. Additionally, they also tried methyl 2,3,4-tri-*O*-trimethylsilyl- α -D-mannopyranoside as a starting material to achieve the product in three steps and obtained the highest yield, around 76% [5]. But chemical synthesis is not economical as it requires a lot of hydroxyl ions as protecting agents, as well as some toxic reagents used as solvents or catalysts in the reaction. It also sometimes requires extremely low temperatures. Finally, the total yield is not efficient.

Additionally, M6P can also be synthesized by enzymatic phosphorylation of mannose using ATP as a phosphate donor. Enzymatically catalyzed reaction conditions are mild, specific to the substrate, and few by-products are formed, and it is easy to remove and to purify the required product, which is difficult with chemical catalysts that require more steps for separation and purification. But it has certain disadvantages as well, such as the requirement of ATP, which is expensive and has poor stability.

In this study, M6P was synthesized using a hexokinase enzyme which can use inorganic polyphosphate (poly(P)) as a phosphate donor. Polyphosphate (poly(P)) is an inorganic phosphate polymer, which is polymerized from a plurality of phosphate residues. These residues are linked to high-energy phosphate bonds in ATP, so they are poly-polymerized. Phosphate can directly or indirectly participate in the phosphorylation of biomolecules [6]. Mukai et al. studied a strain of *Arthrobacter* sp., and found that the bacterium has a polyphosphate-dependent kinase that not only phosphorylates glucose but also phosphorylates mannose. They call this kinase polyphosphate/ATP-dependent grape mannose kinase (GMK) [7].

Previously, our group reported the glucomannokinase from *Mycobacterium phlei* [8] for synthesis of M6P. The results revealed that it was possible to use glucomannokinase for M6P synthesis using inorganic polyphosphate as a donor, however, the conversion was not efficient. In this study, the polyphosphate-dependent mannose kinase (*Arthrobacter* sp. KM) showed significantly improved conversion efficiency under optimized conditions, reaching nearly 100%.

The enzyme used in the catalytic reaction is obtained by fermentation of genetically engineered bacteria and is a polyphosphate-dependent kinase which can synthesize M6P by utilizing inorganic polyphosphate [8]. This method is cost effective as it can replace expensive ATP and uses mild enzyme-catalyzed reactions which is beneficial to industries. This study explores the influence of different factors on the small-scale enzyme-catalyzed reaction, and then performs a simulation prediction analysis using the response surface method (RSM) to obtain the optimal parameters. The response surface method is an optimization method for comprehensive experimental design and mathematical modeling. By testing the representative local points, the regression fits both the functional relationship between the factors in the global scope and the results, and obtains the optimal level of each factor [9]. This study lays a foundation for industrialization of the production process.

2. Results and Discussion

2.1. Construction of Heterologous Expression Vector and Expression of Protein

The protein solution obtained in Section 3.3. was subjected to 12% SDS-PAGE gel protein electrophoresis, and the molecular weight was used as a reference for the standard protein. The results are shown in Figure 1. Compared with the marker, there was an overexpressed band near 30 kDa, which had the same molecular weight as the target protein, indicating that the target protein overexpression was successful.

2.2. Enzyme Catalytic Reaction Verification

In this study thin layer chromatography was used to qualitatively detect the product. The reaction system is shown in Section 3.4. The reaction time was eight hours, and 1 μ L of mannose standard (4% w/v), M6P standard (4% w/v), and reaction solution were spotted on a silica gel plate, stained with vanillin (0.5% w/v), and developed at 105 °C. The results are shown in Figure 2. The reaction solution of the crude enzyme contained mannose-6-phosphate, which indicated that the protease solution expressed by *Escherichia coli* was catalyzed when the 17 gene was transferred, and the enzyme-catalyzed reaction was successful. The chromogenic spots of mannose can be seen at the front of the mannose-6-phosphate spot (Lane 3), indicating that the enzyme catalyzed the reaction and did not react completely, but the substrate spots were small, indicating that the conversion efficiency was high.

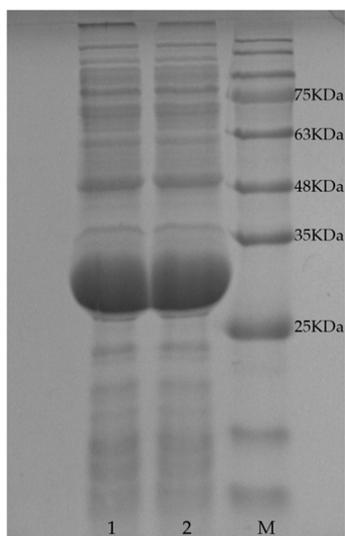


Figure 1. SDS—PAGE results. The protein of about 30 kDa was the result of enzyme expression in *E. coli*. Lane1 and lane2, which have overexpression bands (target protein), are both protein electrophoresis bands of recombinant *E. coli*. Lane M is the marker.



Figure 2. Qualitative detection of mannose-6-phosphate (M6P) by thin layer chromatography. Lane 1 is the result of the mannose standard, lane 2 is the result of the M6P standard, and lane 3 is the result of the reaction solution.

2.3. Optimization of Conditions

2.3.1. Optimization of Reaction Time

For the catalytic reaction of this enzyme, the conversion efficiency over 24 h was selected for measurement, and a graph of conversion vs. time was obtained. It can be seen from Figure 3 that in the first hour, the reaction rate was faster and the conversion efficiency reached 57.14%. But after 8 h of reaction, the conversion efficiency of the reaction gradually became flat, reaching 82.18% at 12 h. After 24 h, the conversion efficiency reached 92.09%. Overall, this polyphosphate-dependent kinase had a good catalytic effect on the synthesis of M6P. According to the curve, the reaction end point was 12 h after the single factor variable experiment began.

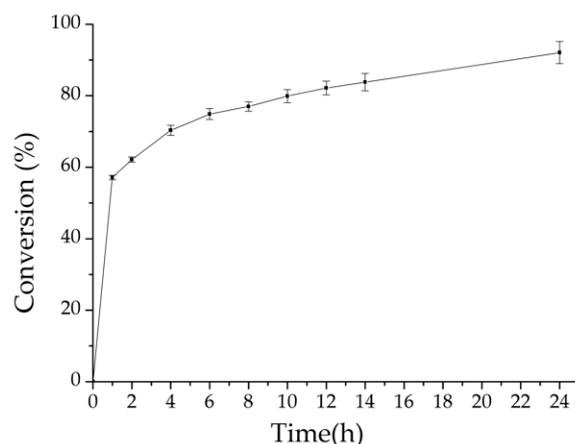


Figure 3. Reaction time course of M6P with polyphosphate-dependent mannose kinase, mannose (20 g/L), $(\text{NaPO}_3)_6$ (60 g/L), and MgCl_2 (5 mM) at 30 °C at pH 8.5. The reaction rate was very fast in the first hour, then slowly decreased, and the conversion efficiency reached 92.09% at 24 h.

2.3.2. Optimization of Temperature

There were five levels of experiments designed for the temperature factor. From Figure 4, we can see that when the conditions were extreme, such as low temperature (10 °C) or high temperature (50 °C), the enzyme showed limited catalytic activity. The conversion was 59.07% and 64.17% efficient, respectively. Based on the experimental results, we speculated that extreme temperatures could partially inactivate the enzyme. Mai et al. found that polyphosphate kinase had the highest enzymatic activity at around 30 °C [10]. The reaction was slightly inhibited when the temperature was 20 °C and 40 °C. The highest conversion was seen at 30 °C, reaching 85.46% conversion efficiency.

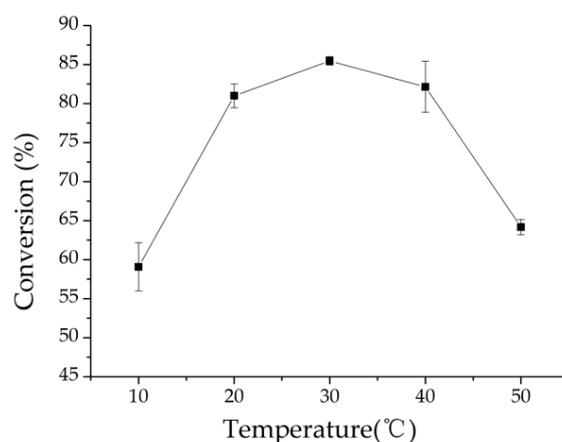


Figure 4. Optimization of temperature in terms of conversion of mannose with polyphosphate-dependent mannose kinase, mannose (20 g/L), $(\text{NaPO}_3)_6$ (60 g/L), and MgCl_2 (5 mM) at pH 8.5. Extreme temperatures would inhibit the reaction, and the highest conversion efficiency was 85.46% at 30 °C.

2.3.3. Optimization of Mg^{2+} Content

Metal ions are critical for polyphosphate-dependent kinase-catalyzed phosphorylation. Mg^{2+} is a cofactor that maintains the activity of the kinase. Sun et al. used molecular dynamics simulations to analyze the role of Mg^{2+} in the phosphorylation of kinases. It was found that Mg^{2+} stabilizes the conformation of substrates and ATP and provides the possibility for phosphorylation [11]. During phosphorylation, it forms a stable complex with ATP and stabilizes the high-energy phosphate bond [12]. In this study, different concentrations of Mg^{2+} were investigated and the conversion

after 12 h of reactions were measured to optimize the maximum product. As it can be seen from Figure 5, when the concentration of Mg^{2+} was less than 10 mM, the reaction conversion was not very high; the conversion efficiency was only 61.23% when the concentration of Mg^{2+} was 1 mM. When it increased to 10 mM of Mg^{2+} , the conversion efficiency jumped to 94.79%. When Mg^{2+} was added in excess, such as 250 mM, the conversion efficiency raised by just 4% and reached 98.07%. It can be seen that between 10 mM and 20 mM of Mg^{2+} is preferable.

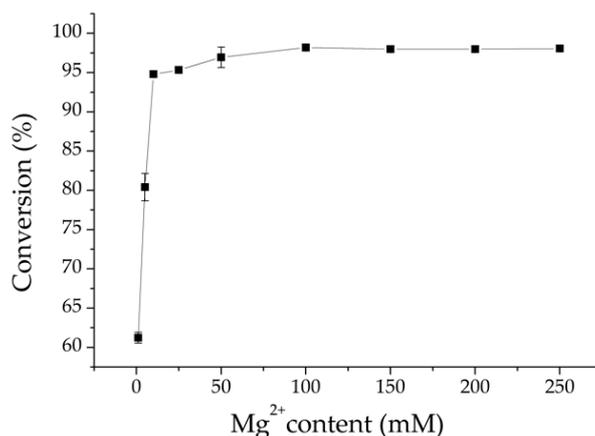


Figure 5. Optimization of Mg^{2+} content in terms of conversion of mannose with polyphosphate-dependent mannose kinase, mannose (20 g/L), and $(NaPO_3)_6$ (60 g/L) at 30 °C at pH 8.5. The effect of Mg^{2+} content on the conversion was mainly at concentrations below 10 mM. When the concentration was more than 10 mM, the conversion efficiency was above 95%, and the change with Mg^{2+} content was not significant.

2.3.4. Optimization of Substrate Addition

Under the same reaction system, different amounts of substrate were investigated to observe any effects on conversion. It can be seen from Figure 6 the reaction conversion efficiency (12 h) decreased when the amount of substrate added increased. At 1% of substrate the conversion efficiency reached 98.15%, but it plummeted to 56.12% when the substrate concentration was raised by 5%.

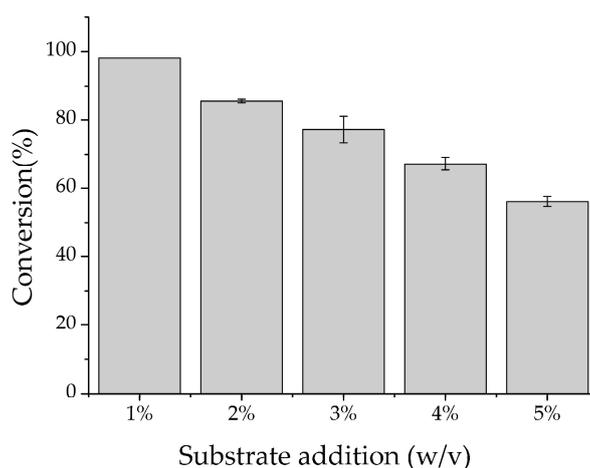


Figure 6. Optimization of substrate addition in terms of conversion of mannose with polyphosphate-dependent mannose kinase, and $MgCl_2$ (5 mM) at 30 °C at pH 8.5. As the amount of substrate increased, the conversion gradually decreased.

Concurrently, the effect of different substrate concentrations on the amount of product produced was also observed over 12 h. As shown in Figure 7, although the 1% group had a high conversion

efficiency, it could only consume 9.814 g/L of substrate per unit time, and the efficiency of the reaction was low. Relatively, the 2% and 3% of substrate concentration groups performed better. Although in the 4% and 5% groups, the amount of substrate added corresponded to a substrate consumption of 17.09 g/L, these reactions were not worth considering from a cost point of view. For example, if the amount of substrate added was 4%, the amount of substrate consumed reached 26.9 g/L but the amount of product was only 1.57 times higher and the cost double that of the 2% group. Therefore, the effect of adding 2% and 3% of the substrate was considered to be economical.

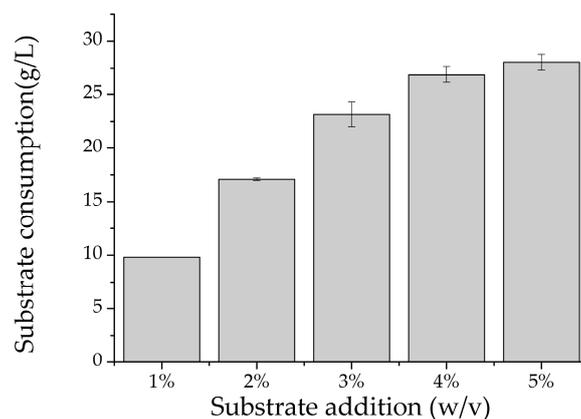


Figure 7. Optimization of substrate addition in terms of substrate consumption with polyphosphate dependent mannose kinase, and MgCl_2 (5 mM) at 30 °C at pH 8.5. As the amount of substrate increased, the substrate consumption also increased.

2.3.5. Optimization of the Substrate Molar Ratio

When investigating the effects of this variable on the conversion efficiency, the amount of fixed mannose added was unchanged, and the amount of sodium hexametaphosphate added was kept at 10, 20, 40, 60, 80, and 100 g/L, respectively. It can be seen from Figure 8 that the conversion efficiency showed a trend of increasing first and then decreasing. Too low or too high concentrations of polyphosphoric acid had inhibitory effects on the reaction. This was probably due to the complex formed between poly(P) and Mg^{2+} , and then the concentration of Mg^{2+} , which could interact with the enzyme molecule, was decreased in the reaction mixture [13]. At 20 g/L, the conversion efficiency reached 99.06%.

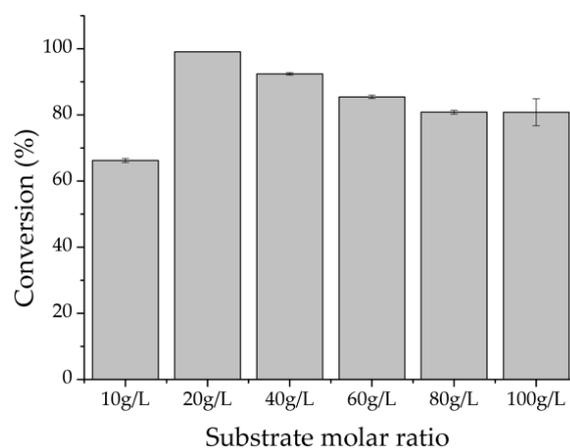


Figure 8. Optimization of the substrate molar ratio in terms of conversion with polyphosphate-dependent mannose kinase, mannose (20 g/L) and MgCl_2 (5 mM) at 30 °C at pH 8.5. As the amount of polyphosphate in the reaction increased, the conversion efficiency first increased and then decreased, and high concentrations of polyphosphate inhibited the reaction.

2.3.6. Optimization of Metal Ions

Bivalent metal ions play a crucial role in the enzymatic reaction of polyphosphate phosphates. When adding the same amount of different types of metal ions, the conversion will be different. Bivalent metal ions can chelate with polyphosphates [14], and this action enables the high energy phosphate bond to stretch better. Kinases are a class of enzymes that transfer a phosphate group from a high energy donor molecule to a specific molecule that requires a bivalent metal ion. Some studies explored the properties of polyphosphate-dependent kinases in cyanobacteria and found that Mg^{2+} was the most suitable bivalent metal ion for this enzyme, followed by Mn^{2+} . When a metal ion such as Cu^{2+} , Ca^{2+} , or Zn^{2+} was added, the enzyme relative activity was less than 15% [15]. All bivalent metal ions were at a concentration of 5 mM. As it can be seen from Figure 9, the conversion efficiency of the Mg^{2+} group was the highest, reaching 85.06%. When other metal ions were added, the conversion efficiency was reduced, and the lowest rate was seen upon adding Cu^{2+} , which was only 33.38%. Not only is the price of these metal ions more expensive than that of Mg^{2+} , but Cu^{2+} , Co^{2+} , and Ni^{2+} were also colored when added to the reaction solution, which may affect the coloration of the product.

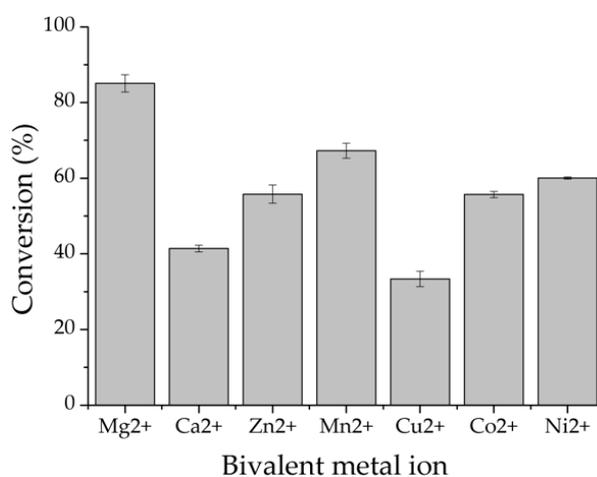


Figure 9. Optimization of bivalent metal ions in terms of conversion efficiency with polyphosphate-dependent mannose kinase, mannose (20 g/L) and $(NaPO_3)_6$ (60 g/L) at 30 °C at pH 8.5. The best effects were found with Mg^{2+} , which is cost-effective and suitable for industrial applications.

2.3.7. Optimization of Various Types of Polyphosphates

The enzyme used in this experiment was a polyphosphate-dependent kinase, so the conversion certainly varied for different phosphate donors. Polyphosphate kinases generally work better on phosphates with longer molecular chains [16]. We selected several polyphosphates for research. The concentration of these polyphosphates was 0.1 M. As shown by Figure 10, when sodium trimetaphosphate, sodium tripolyphosphate, and sodium pyrophosphate were added, the conversion efficiencies were almost zero, indicating that the enzyme almost did not show activity towards such polyphosphates. In contrast, the conversion efficiency could reach 49.15% when ATP was added.

When Liu et al. studied the production of glutathione by polyphosphate-dependent kinase, they used polyphosphate kinase (PPK) and cost-effective phosphate to achieve ATP regeneration. The PPK2 from *C. glutamicum* could utilize hexametaphosphate as a phosphate donor to generate ATP from ADP in vitro. According to the Lineweaver–Burk plots, the K_M value for PPK2 for hexametaphosphate was 29.39 mM and V_{max} was 4.82 $\mu\text{mol}/\text{min}/\text{mg}$. These studies proved that the enzyme is highly selective for hexametaphosphate [17].

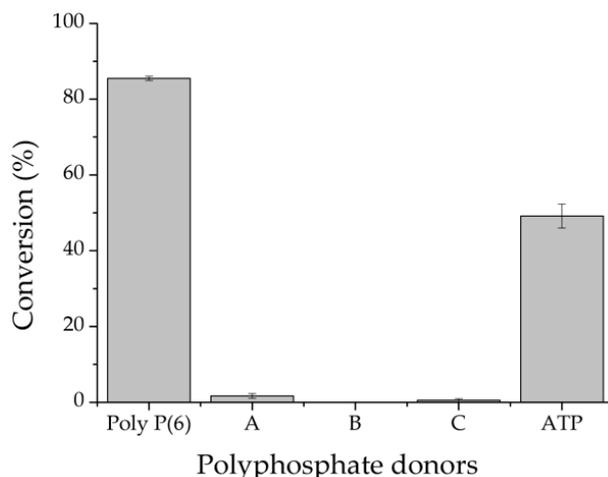


Figure 10. Optimization of polyphosphate donors in terms of conversion efficiency with polyphosphate-dependent mannose kinase, mannose (20 g/L), at 30 °C at pH 8.5. A, B, and C represent sodium trimetaphosphate, sodium tripolyphosphate, and sodium pyrophosphate, respectively. This enzyme was more selective for polyphosphates than ATP and was not selective for A, B, and C.

2.3.8. Determination of Kinetic Parameters

When we chose sodium trimetaphosphate, sodium tripolyphosphate, and sodium pyrophosphate as the phosphate donors, the conversion efficiency was nearly 0%. So, when exploring the kinetic parameters, we only chose to explore the kinetic parameters of ATP and sodium hexametaphosphate. Kinetic parameters of polyphosphate-dependent mannose kinase are shown in Table 1.

Table 1. Kinetic parameters of polyphosphate-dependent mannose kinase.

	ATP	Poly(P)
V_{max} (μmol/min)	0.21	0.14
K_{cat} (s⁻¹)	150.17	201.41
K_M (μM)	4.62	1.7

As can be seen from the data in the table, the K_M of ATP was larger than that of (NaPO₃)₆, indicating that the selectivity to (NaPO₃)₆ was greater than that of ATP for this enzyme.

2.4. Response Surface Method

First, we selected three parameters for analysis, namely Mg²⁺ (A), substrate molar ratio (B), and temperature (C), in a three-factor three-level table. This was shown in Table 2. The dependent variable was the conversion efficiency.

Table 2. Three-factor three-level table.

	−1	0	1
Mg²⁺ content (mM)	1	5	10
Substrate molar ratio (g/L)	20	60	100
Temperature (°C)	15	30	45

The test form was generated and the experiment was performed based on the form. The corresponding conversion efficiency and analysis were recorded in the form. The experiments and results were shown in Table 3. ANOVA consists of classifying and cross classifying statistical results

and testing whether the means of a specified classification differ significantly. This was carried out in this study by Fisher's statistical test for the analysis of variance. The F-value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model [18].

Table 3. Box–Behnken design (BBD) experimental design table and data.

Experiment Code	Treatment	Mg ²⁺ Content (mM)	Substrate Molar Ratio (g/L)	Temperature (°C)	Conversion (%)
16	1	0	0	0	85.58
6	2	1	0	−1	77.55
3	3	−1	1	0	45.225
11	4	0	−1	1	98.75
8	5	1	0	1	96.7
1	6	−1	−1	0	70.3
10	7	0	1	−1	54.55
5	8	−1	0	−1	32.9
4	9	1	1	0	83.6
13	10	0	0	0	85.94
2	11	1	−1	0	99.17
12	12	0	1	1	78.75
15	13	0	0	0	84.87
9	14	0	−1	−1	93.275
14	15	0	0	0	85.58
17	16	0	0	0	85.94
7	17	−1	0	1	40.55

According to ANOVA, the most suitable quadratic polynomial model corresponding to the enzyme catalytic reaction is as follows:

$$\text{Conversion (\%)} = 85.58 + 21.01 \times A + 12.42 \times B + 7.06 \times C + 2.38 \times AB + 2.88 \times AC + 4.68 \times BC - 15.21 \times A^2 + 4.20 \times B^2 - 8.45 \times C^2$$

In the equation, A represents the Mg²⁺ concentration, B represents the substrate molar ratio, and C represents the temperature. The model F-value was 28.31, which implied that this model was significant. There was only a 0.1% chance that a “model F-value” this large could occur due to noise. Values of “probability > F” less than 0.0500 indicated that the model terms were significant. In this case the model terms A, B, C, A², and C² were found to be significant.

Figure 11 shows the effect of the interaction of Mg²⁺ content and substrate molar ratio on conversion efficiency. It was found that the portion of samples having a high conversion efficiency were concentrated at a position where the amount of Mg²⁺ added was high and the amount of polyphosphate added was low. This was consistent with the results of the single factor variable. Both of these were key factors influencing the experiment. Mg²⁺ content was a key factor affecting the experiment, as it stabilizes the substrate and high-energy phosphate bond. A suitable magnesium ion concentration made the conversion rate reach 99% or more, and the excessive sodium hexametaphosphate addition also inhibited the reaction. The amount of polyphosphate added (substrate molar ratio) was also critical. The mutual influence of the two constitutes the 3D map.

It can be seen from Figure 12 that low conversion efficiency was observed at low temperatures and low concentrations of Mg²⁺, while high conversion efficiency was noticed at high Mg²⁺ concentrations and when the temperature was 30 °C. Mg²⁺ content was a key factor of conversion efficiency, and its effects on the experimental results were obvious.

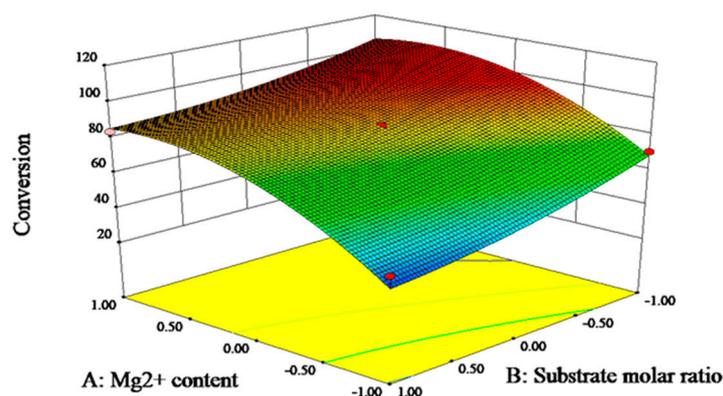


Figure 11. Mg²⁺ content and substrate molar ratio affect the conversion efficiency of the enzyme catalytic reaction. For the substrate molar ratio axis, “−1–1” represents the content of sodium hexametaphosphate from 20 g/L to 100 g/L. For the Mg²⁺ content axis, “−1–1” represents Mg²⁺ content from 1 mM to 10 mM.

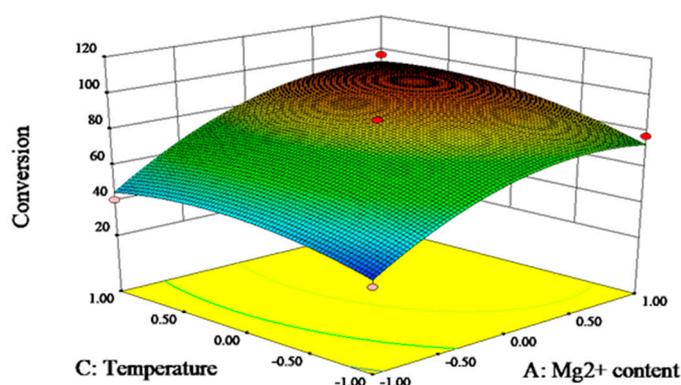


Figure 12. Temperature and magnesium ion concentration affect the conversion efficiency of enzyme catalytic reactions. For the temperature axis, “−1–1” represents temperatures from 15 °C to 45 °C. For the Mg²⁺ content axis, “−1–1” represents Mg²⁺ content from 1 mM to 10 mM.

It can be seen from Figure 13 that the low conversion efficiency was mainly concentrated in the low temperatures and high polyphosphate concentrations. The highest conversion can be seen from the contour line when the added polyphosphate content was around 20 g/L, and the temperature was around 30 °C. In both cases, the amount of polyphosphate added (substrate molar ratio) was a more significant factor than temperature, and the effect on the experimental results was more significant.

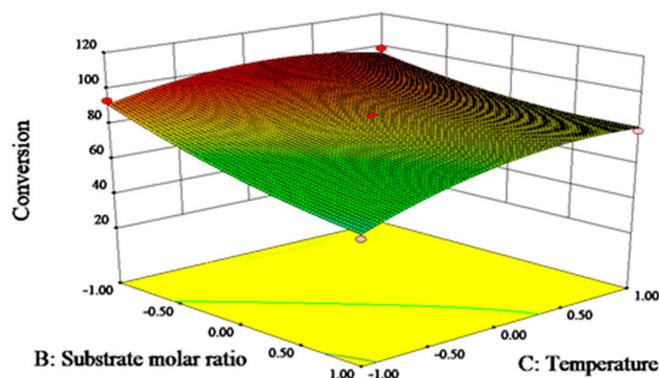


Figure 13. Temperature and the substrate molar ratio affect the conversion efficiency of the enzyme catalytic reaction. For the temperature axis, “−1–1” represents temperatures from 15 °C to 45 °C. For the substrate molar ratio axis, “−1–1” represents the content of sodium hexametaphosphate from 20 g/L to 100 g/L.

2.5. Large Scale Synthesis of M6P

A 200 mL reaction was carried out in a stir reactor for the up-scaled synthesis of mannose-6-phosphate (M6P) in order to prepare for industrial applications, as shown in Section 3.7. Completed conversion was observed within a one hour reaction time. According to optimized reaction parameters, more Mg^{2+} facilitates the stabilization of the conformation of the substrates and phosphate donors, as well as the reaction rate. At the same time, an appropriate molar ratio of substrates eliminates the inhibition of excess polyphosphates on the reaction. The key point was that the enzyme catalyzes efficiently at the optimum pH value, which significantly improves the reaction rate. The scale-up experiment showed that after optimizing and controlling the pH value, the reaction rate increased significantly, and the substrate was quickly converted into product in a very short time. The result of the scale-up experiment revealed an efficient method for industrialization and had good industrial application prospects.

3. Materials and Methods

3.1. Strains, Plasmids, and Chemicals

Escherichia coli strain BL21(DE3) (used as the host for expression of protein) and *E. coli* strain Top 10 (used for DNA cloning) were purchased from Tiangen Ltd. (Beijing, China) and from Biomed (Beijing, China), respectively. Expression vector pET28(a)+ was preserved in our laboratory. The gene of mannose kinase (GMK) with accession number AB096174 was synthesized directly onto pET28(a)+ between NdeI and HindIII according to its sequence by Genscript (Suzhou, China) [7]. Plasmid mini kits and gel extraction kits were purchased from Omega Bio-Tek (Norcross, GA, USA). The chemicals for SDS-PAGE were purchased from BioRo Yee Ltd. (Beijing, China).

3.2. Expression of Recombinant Polyphosphate-Dependent Mannose Kinase

3.2.1. Expression Vector Transformation

The constructed expression vectors pET28a-GMK were transformed into BL21(DE3) *E. coli*. Initially, 1 μ L of plasmid was carefully added to the competent cells and was allowed to stand for 30 min on ice, then the mixture was heat shocked at 42 °C for 90 s and immediately transferred to an ice bath and kept on it for another 4–5 min. Subsequently 500 μ L of sterile LB was added and mixed. Then it was kept at 37 °C, 160–170 rpm in a shaker for 45 min to resuscitate the cells. Later 50 μ L of the resuscitated cells were taken and spread evenly with a sterile coating rod on an agar plate containing 50 mg/mL kanamycin. The culture dish was inverted and cultured in an incubator at 37 °C for 14 to 16 h until the colony was large enough for testing.

3.2.2. Induced Expression

The transformed monoclonal colonies were inoculated into a 4 mL sterile LB tube supplemented with kanamycin, and shaken at 37 °C, 160–170 rpm for 14 h, and the first-stage seed solution in the test tube was taken and inoculated at a 1% ratio. The amount was inoculated into a 50 mL sterile LB shake flask supplemented with kanamycin, and cultured at 37 °C until the OD₆₀₀ was about 0.6, then 0.4 mM IPTG was added, and intracellular expression was induced at a temperature of 25 °C for 12 h.

3.3. SDS-PAGE Protein Electrophoresis

The cells were collected by centrifugation at a low temperature of 4 °C under a rotation speed of 8000 rpm, and resuspended in a Tris-HCl buffer solution with a pH of 7.5 and a concentration of 0.1 mol/L. The resuspended bacterial solution was disrupted using an ultrasonic cell disruptor at a low temperature of 0 °C. The ultrasonic cell disruption power was 80 W, the cell breaking time was 3 s, the interval was 3 s, and the total cell breaking time was 900 s. The crushed protein solution was centrifuged at a low temperature of 4 °C at 10,000 rpm and the supernatant was stored at –20 °C.

Specifically, the electrophoresis steps were as follows: the electrode buffer was injected into the electrophoresis tank, electrophoresis was performed at a constant pressure of 80 V for 20 min, and then the current was changed to a constant 20 mA until the blue loading buffer front stopped at a distance of 1–2 mm from the bottom of the gel to stop electrophoresis. After the electrophoresis was completed, the gel was stained in the staining solution.

3.4. Enzyme Catalytic Verification

A 20 mL reaction substrate solution was prepared which included 0.4 g of mannose, 1.2 g $(\text{NaPO}_3)_6$, 0.1 mol/L Tris–HCl (pH 8.5), 5 mM MgCl_2 , and 5 mL of crude enzyme solution. The reaction system was placed under the conditions of a temperature of 30 °C and a pH of 8.5 for 8 h. At the end of the reaction, the mannose-6-phosphate in the product formed by the reaction was qualitatively detected by thin layer chromatography (TLC).

3.5. Effects of Various Parameters on Polyphosphate-Dependent Mannose Kinase Activity

We further explored the effects of different parameters on the reaction such as time, temperature, Mg^{2+} addition, substrate addition, substrate molar ratio, bivalent metal ion, and polyphosphate donor on the reaction conversion efficiency.

The mannose content was determined using high performance liquid chromatography (HPLC) to calculate the conversion. The reaction liquid was detected by an American boile organic acid column Aminex (Hercules, CA, USA) HPX-87H. The mobile phase was 5 mM dilute sulfuric acid, the flow rate was 0.6 mL/min, and the column temperature was 65 °C. Signals were detected by the refractive index detector, and the detection temperature was 40 °C.

3.5.1. Effect of Reaction Time

The conversion reaction was measured at 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, and 24 h, and the conversion efficiency was observed with time. The reaction system was 20 mL: 0.4 mL mannose mother liquor (200 g/L pH 8.5), 0.6 mL $(\text{NaPO}_3)_6$ (400 g/L, pH 8.5), 5 mM MgCl_2 , 5 mL of crude enzyme solution, and the reaction system was 0.1 M Tris–HCl (pH 8.5). Each sample was depleted of excess $(\text{NaPO}_3)_6$ with 0.2 mol/L CaCl_2 .

3.5.2. Effect of Temperature

The conversion efficiency of the reaction system at 10, 20, 30, 40, and 50 °C for 12 h was measured. The reaction was 4 mL: 0.4 mL mannose mother liquor (200 g/L, pH 8.5), 0.6 mL $(\text{NaPO}_3)_6$ (400 g/L, pH 8.5), 5 mM MgCl_2 , and 1 mL of crude enzyme solution, and the reaction system was 0.1 M Tris–HCl (pH 8.5). Each sample was depleted of excess $(\text{NaPO}_3)_6$ with 0.2 mol/L CaCl_2 .

3.5.3. Effect of Mg^{2+} Content

The conversion efficiency of the reaction system was measured at 1 mM, 5 mM, 10 mM, 25 mM, 50 mM, 100 mM, 150 mM, 200 mM, and 250 mM MgCl_2 for 12 h. The reaction system was 4 mL: 0.4 mL mannose mother liquor (200 g/L, pH 8.5), 0.6 mL $(\text{NaPO}_3)_6$ (400 g/L, pH 8.5), and 1 mL of crude enzyme solution, and the reaction system was 0.1 M Tris–HCl (pH 8.5). Each sample was depleted of excess $(\text{NaPO}_3)_6$ with 0.2 mol/L CaCl_2 .

3.5.4. Effect of Substrate Addition

The conversion efficiency of the reaction was measured for 12 h under the same reaction system when the amount of the substrate added was 1%, 2%, 3%, 4%, and 5%. The reaction system was 4 mL, and the amount of the substrate was changed according to the experimental conditions. The reaction included 5 mM MgCl_2 and 1 mL of crude enzyme solution, and the reaction system was 0.1 M Tris–HCl (pH 8.5). Each sample was depleted of excess $(\text{NaPO}_3)_6$ with 0.2 mol/L CaCl_2 .

3.5.5. Effect of Substrate Molar Ratio

The amount of mannose added was unchanged, and sodium hexametaphosphate was added at 10, 20, 40, 60, 80, and 100 g/L, and the conversion efficiency after 12 h of reaction time was measured. The reaction system was 4 mL: 0.4 mL mannose mother liquor (200 g/L, pH 8.5) and 1 mL of crude enzyme solution, and the reaction system was 0.1 M Tris-HCl (pH 8.5). Each sample was depleted of excess $(\text{NaPO}_3)_6$ with 0.2 mol/L CaCl_2 .

3.5.6. Effect of Metal Ions

Different 5 mM bivalent metal ions (Mg^{2+} , Ca^{2+} , Zn^{2+} , Mn^{2+} , Cu^{2+} , CO^{2+} , and Ni^{2+}) were added, and the conversion efficiency after 12 h of reaction time was measured. The reaction system was 4 mL: 0.4 mL mannose mother liquor (200 g/L, pH 8.5), 0.6 mL $(\text{NaPO}_3)_6$ (400 g/L, pH 8.5), 1 mL of crude enzyme solution, and the reaction system was 0.1 M Tris-HCl (pH 8.5). Each sample was depleted of excess $(\text{NaPO}_3)_6$ with 0.2 mol/L CaCl_2 .

3.5.7. Effect of Various Types of Polyphosphate

Different polyphosphate donors (sodium trimetaphosphate, sodium tripolyphosphate, sodium pyrophosphate, and ATP) were added and the conversion was measured at 12 h. The reaction system was 4 mL: 0.4 mL mannose mother liquor (200 g/L, pH 8.5), the same molar concentration of polyphosphate mother liquor (pH 8.5), 5 mM MgCl_2 , and 1 mL of crude enzyme solution, and the reaction system was 0.1 M Tris-HCl (pH 8.5). Excess polyphosphate from each sample was removed with 0.2 mol/L CaCl_2 .

3.5.8. Determination of Kinetic Parameters

ATP and polyphosphate were selected as phosphate donors to determine the kinetic parameters of the enzyme. As the affinity of the enzyme to mannose was far less than that of glucose, in order to simplify the kinetics measurement and reduce errors, the selectivity of the enzyme to different phosphoric acid donors was studied using glucose as a substrate. As mentioned above, a phosphate donor was selected to measure the initial reaction rate of the enzyme under different concentrations of phosphate donor, and a double reciprocal curve was drawn.

3.6. Response Surface Method

There are a variety of response surface test design methods, such as Box-Behnken design (BBD) and central composite design (CCD). This study used the BBD model. BBD is a commonly used experimental design method for response surface optimization. It is suitable for optimization experiments with 2 to 5 factors [19]. Each factor takes 3 levels and is coded as $(-1, 0, 1)$. The design table is arranged with 0 as the center point, and +1, -1 are the high and low values corresponding to the cube points, respectively.

After designing the three-factor three-level table, a test plan table was generated, and the experiment was performed according to the table, that is, the global approximation of the output variable (system response) was fitted to replace the real response surface.

3.7. Large Scale Synthesis of M6P

This study carried out an amplification experiment for the synthesis of mannose-6-phosphate (M6P) in a 1 L stir reactor. The reaction system was 200 mL: 4 g mannose (20 g/L), 4 g polyphosphate (20 g/L), 50 mL crude enzyme solution, and 2 mL 1 M MgCl_2 (10 mM) and used 0.1 M Tris-HCl to a constant volume to 200 mL. pH was adjusted with 6 mol/L KOH and the pH value of the reaction system was maintained at pH 8.5, 200 rpm. Conversion efficiency was measured by high performance liquid chromatography (HPLC) and substrates and products were qualitatively detected by TLC.

4. Conclusions

This study successfully demonstrates a cost-effective method for the catalytic synthesis of mannose-6-phosphatase (M6P), which synthesizes M6P by polyphosphate-dependent mannose kinase from *Arthrobacter*. We achieved the heterologous expression of the enzyme in *E. coli*, and optimized the reaction system by single factor experiments and the response surface method (RSM). The conversion efficiency was increased up to 99%. In this study, polyphosphate was used as a donor instead of expensive ATP, which improved the production efficiency and solved the cost problem in mass production. In the 200 mL experiment, the substrates were completely converted into products within an hour, with significantly improved reaction efficiency. This method has potential industrial application prospects. This method of enzyme-catalyzed synthesis is safer than chemical methods and lays the foundation for industrialization, providing a method for low-cost production of M6P.

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