http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (16): 2001-2006, 2008
ISSN 1028-8880

Isomalt Production by Cloning, Purifying and Expressing of the MDH Gene From *Pseudomonas fluorescens* DSM 50106 in Different Strains of *E. coli*

[1]M. Haghighatian, [2]M.R. Mofid, [2]M.K. Nekouei, [1]P. Yaghmaei and [2]A.H. Tafreshi
[1]Science and Research Branch, Islamic Azad University, Iran
[2]Agricultural Biotechnology Research Institute of Isfahan, Pazhuhesh Boulevard, Najafabad Road, Isfahan, Iran

Abstract: A NADH-dependent mannitol dehydrogenase gene (*mtlD*) was cloned from *Pseudomonas fluorescens*, subcloned into an expression vector (pDEST110) and entered into different strains of *E. coli* to compare their protein expression and the enzyme specific activity. Purifications were accomplished by Ni^{2+}-NTA affinity chromatography. Using this approach, the efficiency of purification process significantly increased (up to 90%) so that the purified enzyme gave a sharp single band (55 kDa) in SDS-PAGE. The results showed that among the strains, BL21 (DE3) plysS exhibited the maximum expression level of MDH(mannitol dehydrogenase) (11 mg L^{-1}). Results from activity assay with fructose as substrate also showed that in this strain the specific activity of 63 U mg^{-1} protein monitored for the enzyme, the record not reported before. Resazurin staining also indicated that the enzyme reduced fructose, whereas oxidized other substrates including mannitol, sorbitol and arabitol under optimal assay condition. From HPLC analysis it was showed for the first time that the enzyme could convert substrate isomaltulose to the specific products, GPM and GPS. Interestingly, because of the high specificity of the enzyme for substrate, the method can be used as an alternative approach to substitute non-specific conventional method of isomalt production.

Key words: Isomalt, mannitol, NAD-dependent mannitol dehydrogenase, Ni^{2+}-NTA affinity chromatography, HPLC

INTRODUCTION

Mannitol is an acyclic hexitol and the most abundant sugar alcohol found in nature (Horer *et al*., 2001). It is produced in different organisms such as bacteria, fungi and higher plants (Jennings *et al*., 1998) where it is used as a source of carbon, a storage compound for energy and a reducing equivalent (Voegele *et al*., 2005). It also functions as a compatible solute during osmoregulation to respond to oxidative stress (Stoop and Pharr, 1994; Conde *et al*., 2007). D-mannitol is widely utilized in food and pharmaceutical industries as an excellent bulking agent (Soetaert *et al*., 1999). This product has been preferred by consumers because of its safety for diabetics and its tooth friendly. Mannitol sweetness is about half of sucrose and is not metabolized by human body and therefore considered as a low-calorie sweetener (Ronda *et al*., 2005).

Methodologically, the mass production system of mannitol involves in reducing fructose using Ni^{2+} ion as catalyst in presence of hydrogen gas. Due to the low selectivity of the catalyst, the procedure finally results in equal amounts of sorbitol and mannitol which must be separated through additional stages (Brünker *et al*., 1997). An alternative method is to use microorganisms and their enzymes to produce mannitol without producing any additional sorbitol. This is based on synthesizing mannitol by NAD(P)H-dependent mannitol dehydrogenase (MDH)s belong to dehydrogenase reductases family. Based on sequence analysis data, protein size and coenzyme-binding motifs, the family divided into three groups including as Short-Chain Dehydrogenase Reductase (SDRs), Medium-Chain Dehydrogenase Reductase (MDRs) and Long-Chain Dehydrogenase Reductase (LDRs). The SDRs bear a typical coenzyme-binding site, GXXXGXG and an active site YXXXK (Nidetzky *et al*., 1996). The MDR enzymes are zinc dependent and have the N-terminal coenzyme binding motif, GXGXXG (Otte and Lengeler, 2001). Despite an overall sequence identity as low as 10%, the LDR enzymes have a conserved KXXXXNXXG motif (Lee *et al*., 2003). MDHs from bacteria are considered as members of the LDR family with 66 recognized members (Lee *et al*., 2003).

Corresponding Author: Mohammad Reza Mofid, Agricultural Biotechnology Research Institute of Isfahan, Pazhuhesh Boulevard, Najafabad Road, Isfahan, Iran Tel: +98 0331 2235858 Fax: +98 0331 2234587

Isomalt is also a natural sugar substitute, a sugar alcohol which is primarily used for its sugar-like physical properties. It has only a small impact on blood sugar levels, does not promote tooth decay and has one half the calories of sugar. It also has an effect like dietary fiber in the gut and produces butyric acid and propionic acid that act as antioxidants. It is an odorless, white and crystalline substance containing about 5% water of crystalline. Isomalt has minimal cooling effect lower than many other sugar alcohols. It is unusual as it is a natural sugar alcohol that is produced from beets. Isomalt (6-O-α-D-glucopyranosid-D-fructose) is manufactured in a two-stage process in which sugar (Saccharose) is first transformed into isomaltulose, a reducing disaccharide that is then hydrogenated using a Raney metal catalytic converter. The final product -isomalt- is an equimolar composition of 6-O-α-D-glucopyranosid-D-sorbitol (1,6-GPS) and 1-O-α-D-glucopyranosid-mannitol-dihydrate (1,1-GPM-dihydrate). The molecular changes that occur during these steps make isomalt to have more chemical and enzymatical stabilities than sucrose. Isomalt stability is the reason for many of its health benefits and the large variety of improved products (Ndindayino *et al*., 2002).

In this study, we cloned a mannitol dehydrogenase gene from *Pseudomonas fluorescens* DSM 50106, subcloned it into an expression vector, expressed the deduced protein in different strains of *E. coli* and purified the enzyme by affinity chromatography to test the best MDH expression system among the strains.

As the other part of this research, we studied the *in vitro* effect of the purified enzyme on a new substrate, isomaltulose for the first time and showed that the enzyme effectively could convert the substrate to isomalt, a favorable sugar substitute.

MATERIALS AND METHODS

Microorganisms and *E. coli* strains: The strains of *E. coli* used in this study were: BL21(DE3)PlysS, JM109, Origami (DE3) and M15 (Table 1). The strains were grown in LB liquid medium or on agar plates with appropriate antibiotics. *Pseudomonas fluorescens* DSM 50106 was grown at 30°C.

Molecular cloning: Total genomic DNA from *Pseudomonas fluorescens* DSM 50106 was extracted according to Sambrook method (Sambrook and Russell, 2001). One pair of primer (Forward: 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GAA ACT GAA TAA GCA GAA -3' and Reverse: 5'-GGG GAC CAC TTT GTA CAA AGC TGG GTC TTA AAC CGG TTT CTT CAG GTG -3') was designed and synthesized by Metabion company (Germany) according to conserved regions of previously reported bacterial LDRs. PCR performed using the following protocol: DNA was denatured at 95°C for 3 min followed by 10 cycles of amplification (95°C for 30 sec, 56°C for 30 sec, 68°C for 2 min), then by 30 cycles (95°C for 30 sec, 58.5°C for 30 sec, 68°C for 2 min) and finally by 4 min at 72°C. GATEWAY® system was used to create the expression vector, *mtlD*-pDEST110 (Fig. 1) according to manufacture's instruction (Invitrogen, USA). The resulting plasmid was entered into *E. coli* strains by electroporation (Bio-Rad, Gene Pulser Xcell™, USA). The resulting plasmid *mtlD*-pDEST encoded an MDH protein by c-terminal His_6 tag.

Table 1: Bacterial strains used in this study

E. coli strains	Genotype
Bl21(DE3)plysS	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3) pLysS(cm^R)
JM109	EndA1 glnV44 thi-1 relA1 gyrA96 recA1 $mcrB^+$ Δ(lac-proAB) e14-[F' traD36 $proAB^+$ $lacI^q$ lacZΔM15] $hsdR17(r_K^- m_K^+)$
Origami(DE3)	Δ(ara–leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL, F'[lac+ lacI q pro] (DE3) gor522::Tn10 trxB (KanR, StrR, TetR)
M15	Nal^s, Str^s, Rif^s, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^+, F^-,$RecA^-$, Uvr^+, Lon^+

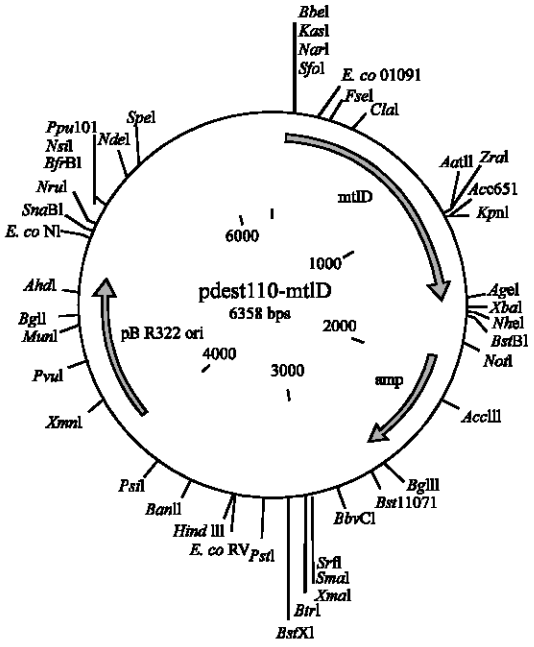

Fig. 1: The genomic map of expression plasmid pDEST110-*mtlD*. The gene was located under the control of T_7 promoter. The deduced protein contained His-tags at C-terminal

Expression of MDH: Ten milliliter of an overnight culture of each transformed *E. coli* strains was transferred into 1 L of LB broth supplemented with appropriate antibiotics.

The cultures were shacked in 250 rpm at 37°C until the OD_{600} reached to about 0.5. Expression of the recombinant *mtlD* gene was induced by addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM. Incubation was continued for a period of 4 h, after which cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C (Eppendorf 5010 R, Hamburg, Germany) followed by washing twice with buffer A (5 mM K_3PO_4, 300 mM NaCl, 10% Glycerol, pH 7.3). The pellet resuspended in 10 mL of the additional buffer A. Bacterial disruption performed by adding 2 mg mL^{-1} lysozyme (Fluka) and the cell debris was removed by centrifugation at 14000 rpm for 1 h in 4°C.

Purification of recombinant protein: All steps were performed at 4°C. Purification of mannitol 2-dehydrogenase protein from supernatant was performed by Fast Protein Liquid Chromatograph (FPLC) system (Sykam, Germany). The system consisted of S 2100 solvent delivery system, S 5111 injektor valve bracket, S 3210 UV/visible detector and Ni^{2+}-NTA affinity chromatography column (Amersham Hi-Trap 1 mL). The column was first equilibrated with buffer A and then the whole extract was loaded on the column. The elution was performed by a linear gradient condition from a mixture of 100% buffer A to 100% buffer B (buffer A +500 mM imidazol) within 39 min. Flow rate was 1 mL min^{-1} and absorbance at 280 nm were measured. The fraction loaded on acrylamide gel (12%) as previously described by Lammlie. The MDH-containing fractions were pooled and dialyzed against 50 mM assay buffer (K_2HPO_4 pH 7.3). After dialysis, the protein solution was concentrated to 1 mL with an ultra-centrifugation filter (Amicon) and used as purified enzyme during the following experiments. Protein concentration was measured by the method of Bradford with bovine serum albumin (Sigma) as standard and stored in 10% glycerol (v/v) at -80°C.

MDH activity assay: The activity of MDH was determined spectrophotometrically by monitoring the change in A_{340} upon oxidation or reduction of NAD(H) at 25°C. The reaction mixture (1 mL) consisted of 2 mM NADH, 100 mM fructose and 20 μL enzyme solution in 50 mM potassium phosphate buffer, pH 7.3. The reaction was started by adding fructose and the mixture allowed standing for 30 sec. Reverse (oxidation) activity of the enzyme also assayed via determination of increasing absorption in A_{340}. The activity was finally expressed as unit per milligram of protein. Effect of the enzyme on different polyol sugars were also showed spectrophotometrically on the conditions explained earlier.

Activity staining: MDH activity was monitored during MDH purification using Resazurin staining described by Perrot *et al.* (2003) with some modifications. Briefly, The staining mixtures consisted of 100 mM fructose, 50 mM K_2HPO_4, 15% agarose, 100 μL purified enzyme and 2 mM NADH were incubated at 25°C for 30 min.

HPLC and TLC: HPLC (High Performance Liquid Chromatography) and TLC (Thin Layer Chromatography) were used to monitor the enzyme activity, control assays in the absence of enzyme or sugar were performed in parallel. Sugar quantification was carried out by injecting the samples (20 μL) to HPLC (Sykam, Germany) equipped with a CROMRESIN Ca^{++}, IEX, 8 μm column (300x, 8 mm). Elution was performed by a mobile phase of 100% double distilled water (HPLC-grade) within 30 min at 70°C at a flow rate of 0.5 mL min^{-1}. Effluents was monitored by using a refractive index detector (Sykam, S 3580, Germany). Integrations were carried out with the HPLC software package (Chromstar 6.3).

Silica-gel Thin Layer Chromatography (TLC) performed to determine the presence of sugar polyols. Four microliter of each sample spotted on a thin layer chromatography alufoil plate (Silica Gel 60 F_{254}, Merck) and developed with ethylacetate/acetic acid/methanol/water (60:15:15:10) as the mobile phase. A solution of 0.5% $KMnO_4$ in 1 M NaOH was sprayed on TLC sheet to stain the sugars. The sheet was heated at 95°C for 3 min before visualization. Each standard sample (fructose and mannitol) was prepared from Sigma, dissolved in ddH_2O up to 1 M (final concentration) and loaded (2 μ) on TLC.

RESULTS AND DISCUSSION

Molecular cloning: Five clones were chosen according to growth on the ampicillin agar plate and used for gene sequencing. All the sequencing results were the same. This similarity attributes to the use of AccuPrime™ *Pfx* DNA polymerase with a high specificity and fidelity causing no point mutation. This sequence had 100% similarity with the results of Brünker *et al.* (1997) (Fig. 2).

Overexpression of MDH in different strains of *E. coli*: The results from cloning and sequencing showed that we successfully obtained a MDH gene from *P. fluorescens*. This fragment had a high identity to another gene has been isolated by Bruncker *et al.* (1997). In order to express the enzyme in different strains of *E. coli*, the resulting construct was subcloned in a destination vector, pDEST110 to create *mtlD*-pDEST110. Different strains of *E. coli* transformed with the plasmid *mtlD*-pDEST110 to

CATTGATAGAGTTATTTTACCACTCCCTATatgaaactgaataagcagaacctcacccagctgg
cgcccgaagtgaaattgccagcctatacgcttgccgacacacgccagggcatcgcccatatcggcgtcggcggcttccat
cgcgcgcaccaggcgtattacacgatgcgctgatgaataccggcgagggcctggactggagcatctgcggcgttggcc
tgcgcagcgaggacgcaaggcccgcgatgacctggccggccaggactacctgttcaccctgtacgaactgggcgaca
ccgacgacacccgaagtgcgcgtgatcggctcgatcagcgacatgctgctggccgaagacagcgcccaggcattgatcg
ataaactggccagcccccgagattcgcatcgtctcgctgaccatcaccgaaggcggctactgcatcgacgacagcaacgg
cgaattcatggcccacttgccgcagatccagcacgacctggctcatccgtcgtcgcccaaaaaccgtgttcggctttatctgc
gcggcattgacccagcgccgcgcggccggcatcccggcgtttaccgtgatgtcctgcgataaacctgcccccacaatggcg
ctgtcacgcgcaaggcactgctggcgttcgccgcccctgcacaacgccgagctgcatgactggatcaaggcccatgtgag
cttcccgaacgccatggtcgaccgcatcacgccgatgaccagcacccgcccacccgcctgcaactgcacgatgaacacggc
atcgacgatgcctggccagttgtttgcgaacccttgtgcagtgggtactggaagacaaattcgtcaacggccgcccggcg
tgggaaaaggttggcgtgcagttcaccgacgatgtgacacccctatgaagagatgaagatcggcttgctcaacggcagccca
cctggccctgacctacctgggttttctcaagggctatcggtttgtgcacgagaccatgaacgacccgctgttcgtggcctaca
tgcgcgcctacatggacctcgacgtcacgccaaacctcgcgccggtaccgggcatcgacctgaccgactacaagcagac
cctggtggaccgcttctccaaccaggcgattgccgaccagttggaacgggtgtgttcggatggctcgtcgaagtttcccaa
gttcaccgtgccgaccatcaaccgcctgattgccgacggccgtgagacgagcgtgcagcactggtcgtcgcggcctgg
gccttgtatttgaagggtgtggatgagaatggcgtgagctacacaatcccgatccgcgccgcgagttctgccaggggctg
gtgagtgacgatgcactgatcagccagcggttgctggcagtggaagagattttcggtacggctattcccaactcgcctgagt
ttgtggcagcgttcgagcggtgctatgggagcctgcgtgataacggcgtcaccactaccctgaagcacctcctgaagaaa
ccggtttaaCAGTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAA

Fig. 2: Sequencing of *mtlD* gene cloned in this study: small and capital letters show the sequence of cloned gene and primer, respectively

select the best strain in terms of MDH expression. The results showed that the treatment of bacteria for 4 h with IPTG at 37°C obtained the highest expression level of the enzyme during the experiments and that among the strains, BL21 (DE3) plysS exhibited the maximum expression level (11 mg L^{-1}). Therefore, this genotype was confirmed to be the best strain for expression of MDH in both laboratory and industrial applications.

Purification of recombinant MDH: Affinity chromatography with Ni^{2+}-NTA resins has been used for the purification of a variety of proteins (Whitford, 2007). This method is one of the most important techniques for high purification of proteins, especially those of analytical and pharmaceutical interest. Principally, proteins with a stretch of histidine residues at N, C or both terminals have a high affinity to Ni^{2+} matrix. The method was applied previously to purify the MDH protein with six histidine tag at the C-terminal (Brünker *et al*., 1997). Methodologically, MDH was bound to Ni^{2+} ions and then eluted with buffer B. The protein contamination was eluted by loading resin with buffer A containing imidazol. It was showed that approximately pure mannitol dehydrogenase was eluted using 335 mM imidazol (Fig. 3). Practically, we did not use any precipitation step in order to protect the minor deals of MDH protein. We also loaded supernatant on the column twice and then kept the column on ice for 30 min. This approach significantly increased the efficiency of purification process (up to 90%). As it is showed in Fig. 3, the purified enzyme gave a single band (55 kDa) in SDS-PAGE.

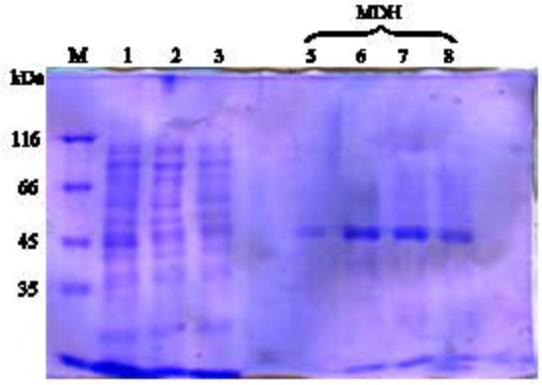

Fig. 3: SDS-PAGE of MDH after affinity chromatography. Extracted Protein fractions of transformed *E. coli* B121(DE3)plysS derived by chromatography, loaded on a 12% SDS-PAGE. M: Molecular weight standard was obtained from Fermentase company. Lane 1, 2: crude extract, Lane 3: crude extract after loading on Ni^{2+}-NTA column, Lanes 5-8: Pure eluted mannitol dehydrogenase = 55 kDa

Assay activity: The activity of the purified enzyme was determined from A_{340} using a spectrophotometer. With Fructose as a substrate, the specific activity of 63 U mg^{-1} protein was monitored for the enzyme in strain BL21(DE3) plysS. According to this data, this value was higher than any records reported previously (Brünker *et al*., 1997). Comparing to the values yielded in other strains, this high enzymatic activity was significantly related to the higher expression of the protein in strain BL21 (DE3) plysS.

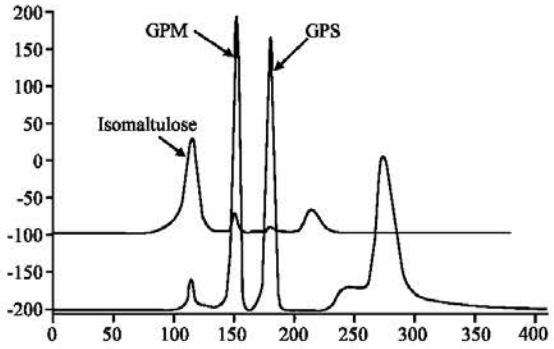

Fig. 4: HPLC chromatograms; A: chromatogram of MDH mixture reaction in the presence of isomaltulose and NADH. Appeared peaks (GPM and GPS) are two necessary fractions of sugar substitute isomalt. B: chromatogram of isomalt standard (standard retention times: isomaltulose 12.1 min, 1,1 GPM 15.1 min, 1,6 GPS 17.4 min)

Therefore, it is concluded again that this strain has the most potential for producing of the enzyme among the strains we used and as a result it can be used in large scale protein purification. Resazurin staining was performed to confirm the reductive or oxidative state of the reaction. When the reaction was reductive, the reaction mixture turned to white, whereas in the case of oxidative reactions there was no change in color of mixture. The staining finally showed that the enzyme was active in reducing fructose whereas, oxidized other substrates including mannitol, sorbitol and arabitol under optimal assay conditions.

Substrate and product analysis by TLC and HPLC: HPLC analysis showed a significant decrease in fructose concentration in the reaction mixture containing NADH, accompanied by the presence of mannitol detected on TLC (Fig. 5).

To test the possible activity of MDH on isomaltulose, HPLC performed to analysis the presence of the specific products, GPM and GPS. In the reaction mixtures with NADH, substrate or enzyme, two new peaks with the retention characteristics of GPM and GPS was formed in the chromatograms (Fig. 4). This means that MDH accepts its substrate. This was the first time that *in vitro* activity of mannitol 2-dehydrogenase on isomaltulose was demonstrated. Due to the high specificity of MDH in this approach, the technique can be finally used as a new and alternative method for large scale isomalt production instead of non-specific production systems. It was previously claimed that mannitol 2-dehydrogenase from *Pseudomonas fluorescens* DSM 1056 can accept sugars

Fig. 5: Enzyme activity assay figured by Thin Layer Chromatography (TLC), Lane 1: standard mannitol, Lane 2: sample of the enzyme assay with fructose as a substrate, Lane 3: standard of fructose

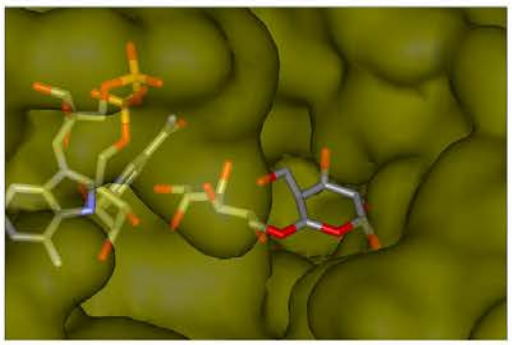

Fig. 6: The MDH structure with NADH molecule on the left as a cofactor and isomaltulose on the right as a substrate illustrated using Weblab Viewer Pro. MDH has enough space for isomaltulose in substrate binding site

with 5-6 carbons as substrate (Kavanagh *et al*., 2002, 2003). In present study, we showed that the enzyme can also act as substrate like isomaltulose with 12-carbon backbone. We suggest that it attributes to the thermodynamic stability of the ligand. In general, sugars in cyclic form in solvent are more stable than linear form. Stability of glucose in cyclic form to linear form is 90:10, whereas stability of cyclic fructose to linear fructose is 70:30. By adding a cyclic form to the linear form, stability of the cyclic form will be increased. By these descriptions it will be appeared that why mannitol 2-dehydrogenase

can affect on isomaltulose. Demonstration of MDH using PDB (Protein Data Bank) site and Weblab Viewer Pro. software showed that MDH in presence of isomaltulose and NADH as substrates had a binding site with enough space for isomaltulose (Fig. 6) not for trehalose which is a trisaccharide.

ACKNOWLEDGMENTS

We are grateful to ABRICI's researchers for their helpful aid during this study. We also express our sincere thanks to M. Shariati of Isfahan University supporting parts of assays.

REFERENCES

Brünker, P., J. Altenbuchner, K.D. Kulbe and R. Mattes, 1997. Cloning, nucleotide sequence and expression of a mannitol dehydrogenase gene from *udomonas fluorescens* 50106 in *Escherichia coli*. Biochem. Biophys. Acta, 1351: 157-167.

Conde, C., P. Silva, A. Agasse, R. Lemoine, S. Delrot, R.M. Tavares and H. Gerós, 2007. Utilization and transport of mannitol in *Olea europaea* and implications for salt stress tolerance. Plant Cell Physiol., 48: 42-53.

Horer, S., J. Stoop, H. Mooibroek, U. Baumann and J. Sassoon, 2001. The crystallographic structure of the mannitol 2-dehydrogenase NADP+ Binary complex from *Agaricus bisporus*. J. Biol. Chem., 276: 27555-27561.

Jennings, D.B., M. Ehrenshaft, D.M. Pharr and J.D. Williamson, 1998. Roles for mannitol and mannitol dehydrogenase in active oxygen-mediated plant defense. J. Plant Biol., 95: 15129-15133.

Kavanagh, K.L., M. Klimacek, B. Nidetzky and D.K. Wilson, 2002. Crystal structure of *Pseudomonas fluorescens* mannitol 2-dehydrogenase binary and ternary complexes. Specificity and catalytic mechanism. J. Biol. Chem., 277: 43433-43442.

Kavanagh, K.L., M. Klimacek, B. Nidetzky and D.K. Wilson, 2003. Crystal structure of *Pseudomonas fluorescens* mannitol 2-dehydrogenase: Evidence for a very divergent long-chain dehydrogenase family. Chem. Biol. Interact., 143-144: 551-558.

Lee, J.K., B.S. Koo, S.Y. Kim and H.H. Hyun, 2003. Purification and characterization of a novel mannitol dehydrogenase from a newly isolated strain of *Candida magnoliae*. Applied Environ. Microbiol., 69: 4438-4447.

Ndindayino, F., C. Vervaet, G. Van Den Mooter and J.P. Remon, 2002. Direct compression and moulding properties of co-extruded isomalt/drug mixtures. Int. J. Pharma, 235: 159-168.

Nidetzky, B., D. Shmidt, A. Weber and K.D. Kulbe, 1996. Simultaneous enzymatic synthesis of mannitol and gluconic acid. II. Process development for a NAD(H)-dependent enzyme system. Biocatal. Biotrans., 14: 47-65.

Otte, S. and J.W. Lengeler, 2001. The mtl genes and the mannitol-1-phosphate dehydrogenase from *Klebsiella pneumoniae* Kay2026. FEMS. Microbiol. Lett., 194: 221-227.

Perrot, S., H. Dutertre-Catella, C. Martin, P. Rat and J.M. Warnet, 2003. Resazurin metabolism assay is a new sensitive alternative test in isolated pig cornea. Toxicol. Sci., 72: 122-139.

Ronda, F., M. Gomez, C.A. Blanco and P.A. Caballero, 2005. Effects of polyols and nondigestible oligosaccharides on the quality of sugar-free sponge cakes. Food Chem., 90: 549-555.

Sambrook and Russell, 2001. Molecular Cloning. 3rd Edn., CSHL Press, New York, ISBN 0-87969-576-5, pp: 4-55.

Soetaert, W., P.T. Vanhooren and E.J. Vandamme, 1999. Production of mannitol by fermentation. Methods Biotechnol., 10: 261-275.

Stoop, J.M.H. and D.M. Pharr, 1994. Mannitol metabolism in celery stressed by excess macronutrients. Plant Physiol., 106: 503-511.

Voegele, R.T., M. Hahn, G. Lohaus, T. Link, I. Heiser and K. Mendgen, 2005. Possible roles for mannitol and mannitol dehydrogenase in the biotrophic plant pathogen *Uromyces fabae*. Plant Physiol., 137: 190-198.

Whitford, D., 2007. Proteins Structure and Function. 1st Edn., Wiley, England, ISBN-13: 978-0471-49893-3, pp: 326-333.