Full Length Research Paper

**In vitro** comparism of the extracellular secretion of inulosucrase enzyme in potential probiotic *Escherichia coli* 16 and BL-21

Prasant Kumar¹,², Sriram Garg Gopalakrishnan¹ and Naresh Kumar, G¹*

¹Molecular Microbial Biochemistry Laboratory, Department of Biochemistry, Faculty of Science, Maharaja Sayajirao University of Baroda, Vadodara-390002, Gujarat, India.
²C.G. Bhakta Institute of Biotechnology, Faculty of Applied Science, UKA Tarsadia University, Bardoli, Surat-394 350, Gujarat, India.

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*Escherichia coli* 16 has potential probiotic properties including antimicrobial activity due to extracellular secretion of colicins E1/1a1b. Inulosucrase (InuJ) enzyme catalyses the polymerization of a fructose moiety of sucrose leading to the formation of fructooligosaccharides. The present investigation compared the activity of InuJ enzymes cloned into pMAL-p2ΔlacI⁰̅ a deletion vector and transformed into *E. coli* 16 and standard strain that is, *E. coli* BL21. Specific activities of InuJ enzyme were estimated in supernatant, periplasm and lysate. Specific activities of InuJ activity in cell lysate without induction of tac promoter with isopropyl thio-β-D-galactoside (IPTG). InuJ activity is mainly present in the periplasm of *E. coli* BL21 whereas in *E. coli* 16, most of the activity is in the supernatant. Superantant of *E. coli* 16 strain also showed good antibacterial activity due to colicin E1/la1b. Colicin E1/1a1b transport system could allow extracellular secretion of InuJ proteins in probiotic *E. coli* 16.

**Key words:** Colicin, extracellular, *E. coli*, fructooligosaccharide, inulosucrase, prebiotic, probiotic.

**INTRODUCTION**

Prebiotics are a category of nutraceutical product that has the ability to promote the growth of specific beneficial gut bacteria (Kelly, 2008). In 2007, Roberfroid defined prebiotics as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gut microflora that confers health benefit”. Fructooligosaccharides (FOS) has been used as prebiotic and is considered as a functional food ingredient (Cherbut, 2002; Fanaro et al., 2005; Bouhnik et al., 2006; Roberfroid, 2007; Paineau et al., 2008). FOS is the common name for fructose oligomers that are mainly composed of 1-kestose (GFS2), 2-nystose (GF3) among others in which fructose units are bound at the β-2, 1 position of sucrose through the transfructosylating enzymes such as glucosyltransferases, fructosyltransferases and inulosucrase (Yun et al., 1996). Inulosucrase has been previously shown to be involved in the synthesis of FOS (Van Hijum et al., 2006). *Escherichia coli*, a Gram negative bacterium is widely used as a host strain for recombinant protein production

*Corresponding author. E-mail: gnaresh_k@yahoo.co.in. Tel: +91-265-2795594. Fax: +91-265-279.

**Abbreviations:** FOS, Fructooligosaccharides; BRP, bacteriocin release proteins; IPTG, isopropylthio-β-D-galactoside; MBP, maltose binding protein.
Table 1. Bacterial strains and plasmids used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>Expression vector, derived from pET15b by insertion of an inulosucrase inuJ gene; Ap'</td>
<td>Anwar et al., 2008</td>
</tr>
<tr>
<td>pET15b- inuj</td>
<td>Ap'</td>
<td></td>
</tr>
<tr>
<td>pMAL-p2ΔlaclQ</td>
<td>deletion of lacI/Q from periplasmic expression vector pMal-p2; Ap'</td>
<td>This study</td>
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<tr>
<td>pMAL-p2ΔlaclQ-</td>
<td>derived from periplasmic expression vector pMAL-p2ΔlaclQ by insertion of inuj; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>inuj</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial</td>
<td>F' ompT hsdSB (R- mB-) gal dcm</td>
<td>Sambrook and Russell, 2001</td>
</tr>
<tr>
<td>E. coli BL21</td>
<td>F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dIacZΔ15Δ(lacZYA- argF)U169, hsdR17(rK- mK+), λ-</td>
<td>Sambrook and Russell, 2001</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>Wild type</td>
<td>Kumar et al., 2009</td>
</tr>
<tr>
<td>E. coli s16</td>
<td>Wild type</td>
<td></td>
</tr>
</tbody>
</table>

in industry. Some natural E. coli strains secrete protein extracellularly but their mechanisms of secretion are not clearly understood, nor are they widely exploited for recombinant protein production and metabolic engineering (Ni and Chen, 2009). In some cases, recombinant proteins directed to the periplasm were found in the medium but the process is not known (Choi and Lee, 2004; Mergulhao et al., 2005). Sommer et al. (2010) constructed a plasmid which contains bacteriocin release proteins (BRP) that allow secretion of recombinant protein from the periplasm into the culture medium (Sommer et al., 2010). It was known that BRP or lysis proteins are responsible for the release of colicins such as A, E1, E2, K, N, U, and Y (Cascales et al., 2007; Singh et al., 2012). Previously, we had reported E. coli 16 isolated from rat fecal matter to possess characteristic such as acid tolerance, antibiotic sensitivity, no pathogenicity and antimicrobial activity against the members of Enterobacteria family. Antimicrobial activity was due colicin E1/1a 1b which is secreted into the culture media in E. coli 16 (Kumar et al., 2009). All these above characteristic attributed E. coli 16 to be a potential probiotic. In this study, we compared the heterologous expression of Inulosucrase from a periplasmic expression vector in the E. coli 16 and E. coli BL-21.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

All the E. coli strains and plasmids used in this study are listed in Table 1. The bacterial strains used in this study were E. coli DH5α, E. coli 16 and E. coli BL-21. Strains of E. coli were grown at 37°C in the Luria-Bertani medium (Hi-Media Laboratories, Mumbai, India). The minimal medium used had the following composition: 12.8 g/l Na2HPO4·7H2O, 3 g/l KH2PO4, 0.5 g/l NaCl, 1 g/l NH4Cl, 3 mg/l CaCl2, 1 mM MgSO4, thiamine and trace elements. Antibiotics were used at the following final concentrations: ampicillin 100 μg ml⁻¹. Plasmid pET15b- inuj was a generous gift from Dr. Dijkhuizen. E. coli DH5α was used for constructing recombinant plasmids. E. coli BL21 and E. coli 16 were used for expressing the proteins under in-vitro condition.

Construction of plasmids: pMAL-p2ΔlacQ and pMAL-p2ΔlacQ-inuj

pMAL-p2 was digested with Mu l/ECORV, end filled and further self ligated, leading to disruption of lacI gene to obtain constitutive pMAL-p2ΔlacQ vector. Confirmation of disrupted clones of pMAL-p2ΔlacQ was done on X-gal plate without isopropylthio-β-galactoside (IPTG). Plasmid pET15b-inuj, containing inulosucrase gene, was digested with NcoI/BamHI to insert the 1.7 kb DNA fragment containing the inuj gene into pMAL-p2ΔlacQ for periplasmic expression. The recombinant plasmid was confirmed by restriction digestion. All the above DNA manipulations were carried out according to the procedures described by Sambrook and Russell (2001). Further confirmation of E. coli harbouring inulosucrase constructs cells were inoculated in M9 media (Hi-Media Laboratories, Mumbai, India) containing 20 mM sucrose as a sole source of carbon.

Transformation of pMAL-p2ΔlacQ and pMAL-p2ΔlacQ-inuj plasmid in both E. coli 16 and E. coli BL-21

The plasmids pMAL-p2ΔlacQ and pMAL-p2ΔlacQ-inuj were independently transformed into E. coli 16 using the CaCl2 method. Similarly, plasmids pMAL-p2ΔlacQ and pMAL-p2ΔlacQ-inuj were also independently transformed into E. coli BL-21 using the CaCl2 method.

Preparation of E. coli cell extracts and Inuj activity assay

E. coli BL21and E. coli 16 harboring pMAL-p2ΔlacQ and pMAL-p2ΔlacQ-inuj constructs were grown overnight and harvested by centrifugation (Heraeus Fresco refrigerated centrifuge, Thermo Scientific, Germany) at 9,200 g for 2 min at 4°C. The cell pellet was washed twice with 50 mM phosphate buffer (pH 7.0) followed by re-suspension in the same buffer. The cells were then subjected to sonication (Branson Sonifier Model 450) for a total period of 1 min at a pulse rate of 15 s in an ice bath, followed by centrifugation at 9,200 g at 4°C for 30 min to remove cell debris. The cell free extract was used for the inulosucrase assays. Periplasmic fraction (Ames et al., 1984) and extracellular fraction were also checked for the presence of the enzyme activity. The initial rate of the inulosucrase reaction was measured at 37°C in 50 mM potassium phosphate buffer (pH 7.0) in the presence of 500 mM sucrose. The enzyme
inulosucrase catalyzes the formation of fructose polymers from sucrose in turn liberating glucose and thus this can be used as an indicator of the enzyme activity. Glucose was estimated using the 3, 5- dinitrosalicylic acid (DNSA) method (Miller et al., 1959).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native gel activity staining of recombinant inulosucrase**

SDS PAGE gel was performed as mentioned by Sambrook and Russell (2001) to detect inulosucrase polypeptide. Samples were mixed with an equal amount of 2X sample buffer (0.125 M Tris HCl [pH 6.8], 1% SDS, 20% glycerol, and incubated at 90°C for 5 min, centrifuged at 10,000 rpm for 20 s, and loaded onto a 12% slab gel. After electrophoresis, the gel was stained with Coomassie blue. Native gels were incubated at ambient temperature in McIlvaine’s buffer (pH 7.0) with 10% sucrose. Formation of FOS within the gel led to white, turbid bands indicating the position of active enzyme. Prolonged incubation caused bursting of the gel due to excessive FOS formation at these sites (Hettwer et al., 1995).

**Statistical analysis**

Statistical evaluation of the data was done by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test and the results were expressed as mean ± SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego, California, USA.

**RESULTS**

**Molecular and phenotypic confirmation of *E. coli* transformants of pMAL-p2ΔlacI and pMAL-p2ΔlacI- InuJ plasmids**

Deletion of repressor binding protein from pMAL-p2 plasmid gave rise to "constitutive phenotype" (Figure 1).

The deleted plasmid clones and the original plasmid clones were plated on X-gal plates without IPTG. It was found that the deletion clone cleave X-gal showed blue colonies while the plasmid control does not cleave X-gal showed white colonies. Deletion of repressor binding protein in pMAL-p2 to form pMAL-p2ΔlacI vector and insertion of inulosucrase gene in pMAL-p2ΔlacI was confirmed by the release of 2.3 kb fragments upon digestion with Sall and BgIII enzymes. Phenotypic confirmation of the clones containing the functional enzyme indeed grew on sucrose without IPTG while the vector controls and wild types did not grow in the presence of sucrose (Figure 2).

**SDS-PAGE and activity of inulosucrase enzymes**

The SDS-PAGE of cell free extracts of *E. coli* BL21 (DE3) containing pET-InuJ showed the presence of 63 kD protein band where as pMAL-p2ΔlacI- InuJ band was seen at 106 kD (Figure 3). This is because InuJ protein is obtained as maltose binding protein (MBP)s-InuJ as a translational fusion protein with a molecular weight of 106 kD. In native gel loaded with the supernatant of *E. coli* 16 harboring pMAL-p2ΔlacI- InuJ incubated in McIlvaine’s buffer (pH 7.0) with 10% sucrose showed clearly a white turbid band indicating the activity of inulosucrase enzymes (Figure 4). Specific activity of inulosucrase enzyme in the supernatant, periplasm and lystate of *E. coli* 16 and BL-21 strain harbouring pMAL-p2ΔlacII-InuJ were monitored (Figure 5a, b and c). In lystate the inulosucrase activities were similar in both the strains harbouring the plasmids. *E. coli* BL21 strain harbouring pMAL-p2ΔlacII-InuJ and pET-InuJ activity were found in the periplasm. While In *E. coli* 16 strain the activity was mostly found in the supernatant.
DISCUSSION

Modernization of diet resulted in a significant increase in the amount of added sugar such as sucrose and fructose, in food products leading to its daily consumption amounting to ≥ 100 g of sugar per day (Basciano et al., 2005). Intake of sweetener above 25% of total energy consumed will cause hypertriglyceridemia and gastrointestinal symptoms (Tappy and Kim-Anne, 2010). High amount of sucrose and fructose in diet ultimately leads to various metabolic disorder such as obesity, diabetes and hypertension.
among others. Complex sugars like FOS on the other hand are beneficial and classified as functional food ingredient (Robertfroid, 2007; Kelly, 2008, 2009). FOS are also known for its antilipogenic effects and are useful in reducing blood glucose level in humans (Delzenne and Kok, 2001).

Interestingly, FOS can be produced from sucrose by the action of enzymes like inulosucrase which break down sucrose and polymerize it into FOS. In this case, we decided to genetically engineer the previously characterized (Kumar et al., 2009) potential probiotic E. coli 16 and E. coli BL-21 to express and secrete InuJ without induction with IPTG. As under in-vivo condition it is not possible to induce the gene by external factors such as IPTG. Similarly we cannot also use such promoter which constitutively expressed and form inclusion bodies. Hence we deleted the repressor protein of pMal-p2, that lead to moderate expression of Inulosucrase without induction with IPTG. Under in-vivo condition, this periplasmic InuJ could now access sucrose in the diet and polymerize it into FOS.

To demonstrate this characteristic we construct the plasmid and transformed into the host such as potential probiotic E. coli 16 and E. coli BL-21 and measured the activity of InuJ by using the DNSA methods to detect free glucose as an indicator of the sucrose hydrolysis (Figure 5). We also checked for direct indication of polymer synthesis using native gel electrophoresis (Figure 4). Surprisingly the heterologous protein even though targeted to the periplasm by the expression vector found its way into the extracellular medium in the case of E. coli 16 but not in E. coli BL-21. Comparisons of specific activity of inulosucrase cloned into periplasmic expression vector, transformed and expressed in E. coli 16 and E. coli BL21 demonstrated that extracellular expression is achieved only in colicin producing microorganism. Various reports suggested that BRP protein is responsible for the release of colicin such as colicin A, E1, E2, K, N, U, and Y into the extracellular medium (Cascales et al., 2007; Sommer et al., 2010; Singh et al., 2012). There are reports that extracellular secretion could be achieved in E. coli strain by co-expression of a lysis-promoting protein (Sommer et al., 2010). E. coli cells having the outer membrane generally do not help to secrete periplasmic proteins into the culture medium.

Here, we have shown a potential probiotic E. coli 16 that expresses E1/1a1b Colicin and its transport system mediates inulosucrase into the culture medium, that leads to the conversion of sucrose into FOS. Extracellular secretion of InuJ enzymes is better than the periplasmic secretion under in-vivo condition. Engineered E. coli16 strain produce colicin and FOS thus its give health benefits; colicin fight against enteropathogens and FOS a prebiotic product activate the beneficial microbes present in gut.

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Specific activity of inulosucrase enzyme in a, Lysate; b, periplasm and c, supernatant of E. coli 16 and BL-21 strain containing (pMAL-p2ΔlacIQ). All values are expressed as mean ± SD (n=4-6) each group and analysis was done using ANOVA. ***p<0.001, **p<0.01 and *p<0.05 compare to E. coli 16.

**Figure 5.** Specific activity of inulosucrase enzyme in a, Lysate; b, periplasm and c, supernatant of E. coli 16 and BL-21 strain containing (pMAL-p2ΔlacIQ). All values are expressed as mean ± SD (n=4-6) each group and analysis was done using ANOVA. ***p<0.001, **p<0.01 and *p<0.05 compare to E. coli 16.
REFERENCES


