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

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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-p2ΔlacI^{Q} 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 were similar in *E. coli* 16 and *E. coli* BL21 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

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Abbreviations: FOS, Fructooligosaccharides; **BRP,** bacteriocin release proteins; **IPTG,** isopropylthio-β-galactoside; **MBP,** maltose binding protein.

Table 1. Bacterial strains and plasmids used.

Strain	Relevant characteristics	Reference/source
Plasmid		
pET15b- inuJ	Expression vector, derived from pET15b by insertion of a inulosucrase inuj gene; Ap^r	Anwar et al.,2008
pMAL-p2ΔlaclQ	deletion of lacIQ from periplasmic expression vector pMal-p2; Ap^r	This study
pMAL-p2ΔlaclQ - inuJ	derived from periplasmic expression vector pMAL-p2Δ$lacl^Q$ by insertion of inuJ ; Ap^r	This study
Bacterial		
E. coli BL21	F′ ompT hsdSB (rB- mB-) gal dcm	Sambrook and Russell, 2001
E. coli DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ-	Sambrook and Russell, 2001
E. coli s16	Wild type	Kumar et al., 2009

in industry. Some natural *E. coli* strains secrete protein extracellulally 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 case, 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 *Enterobacteriacae* 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 Bretani medium (Hi-Media Laboratories,Mumbai, India). The minimal medium used had the following composition: 12.8 g/l $Na_2HPO_4 \cdot 7H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 3 mg/l $CaCl_2$, 1 mM $MgSO_4$, 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* BL21and *E. coli* 16 were used for expressing the proteins under *in-vitro* condition.

Construction of plasmids: pMAL-p2Δ$lacl^Q$ and pMAL-p2Δ$lacl^Q$-*InuJ*

pMAL-p2 was digested with Mlu I/EcoRV, end filled and further self ligated, leading to disruption of *$lacI^Q$* gene to obtain constitutive pMAL-p2Δ$lacl^Q$ vector. Confirmation of disrupted clones of pMAL-p2Δ$lacl^Q$ 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-p2Δ$lacl^Q$ 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-p2Δ$lacl^Q$ and pMAL-p2Δ$lacl^Q$-*InuJ* plasmid in both *E. coli* 16 and *E. coli* BL-21

The plasmids pMAL-p2Δ$lacl^Q$ and pMAL-p2Δ$lacl^Q$-*InuJ* were independently transformed into *E. coli* 16 using the $CaCl_2$ method. Similarly, plasmids pMAL-p2Δ$lacl^Q$ and pMAL-p2Δ$lacl^Q$ -*InuJ* were also independently transformed into *E. coli* BL-21using the $CaCl_2$ method.

Preparation of *E. coli* cell extracts and *InuJ* activity assay

E. coli BL-21and *E. coli* 16 harboring pMAL-p2Δ$lacl^Q$ and pMAL-p2Δ$lacl^Q$ *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

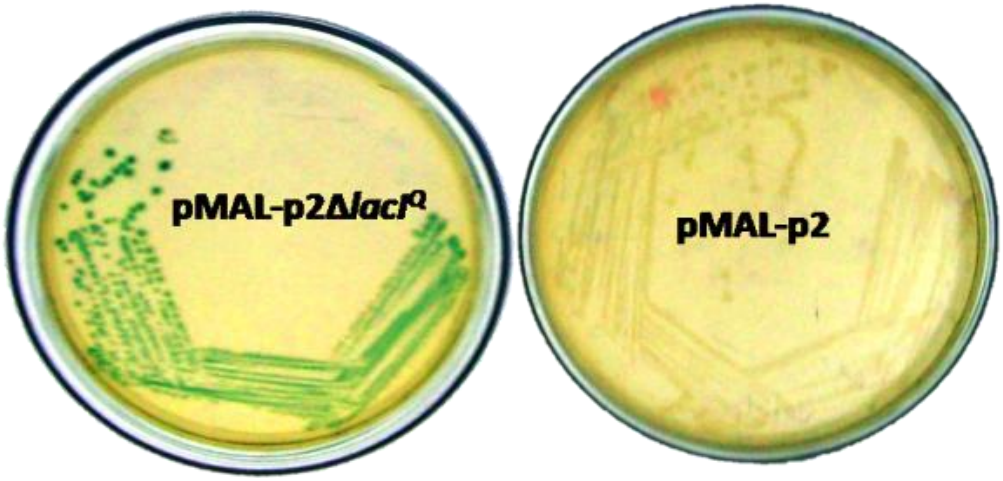

Figure 1. Phenotypic confirmation of *E. coli*16 harbouring pMAL-p2Δ*lacI*^{Q} plasmid on X-gal without addition of IPTG. X-gal breakdown leads to blue colour indicate deletion of lacI^{Q} where as control plasmid without deletion showed colourless.

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 McIllvaine'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-p2Δ*lacI*^{Q} and pMAL-p2Δ*lacI*^{Q}-*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^{Q} vector and insertion of inulosucrase gene in pMAL-p2Δ*lacI*^{Q} was confirmed by the release of 2.3 kb fragments upon digestion with SalI and BglII 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-p2Δ*lacI*^{Q}- *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-p2Δ*lacI*^{Q}- *InuJ* incubated in McIllvaine'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 lysate of *E. coli* 16 and BL-21 strain harbouring pMAL-p2ΔlacI^{Q}-*InuJ* were monitored (Figure 5a, b and c). In lysate the inulosucrase activities were similar in both the strains harbouring the plasmids. *E. coli* BL21strain harbouring pMAL-p2ΔlacI^{Q}-*inuJ* and pET-*InuJ* activity were found in the periplasm. While In *E. coli* 16 strain the activity was mostly found in the supernatant.

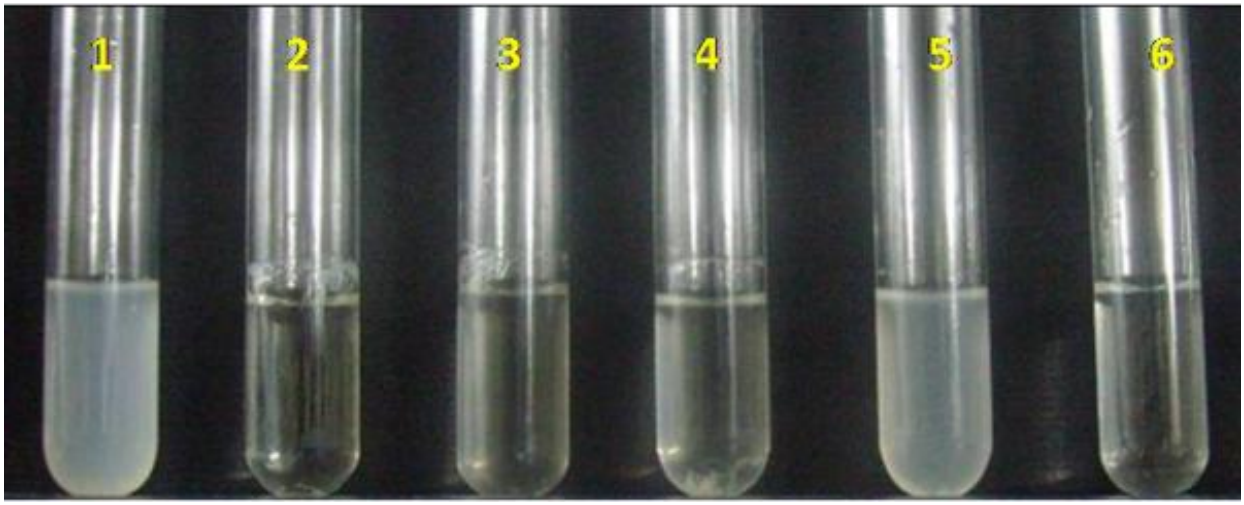

Figure 2. Growth of inulosucrase transformants in presence of M9 medium containing sucrose as a sole carbon source. Lane 1, *E. coli* BL21 (DE3) containing pET-15b-*inuJ* with IPTG; Lane 2, *E. coli* BL21 (DE3) containing pET-15b-*inuJ* without IPTG; Lane 3, *E. coli* BL21; Lane 4, *E. coli* 16 containing pMAL-p2Δ*lacI*^{Q}; Lane 5, *E. coli* 16 containing pMAL-p2Δ*lacI*^{Q} *inuJ*; Lane 6, *E. coli* 16.

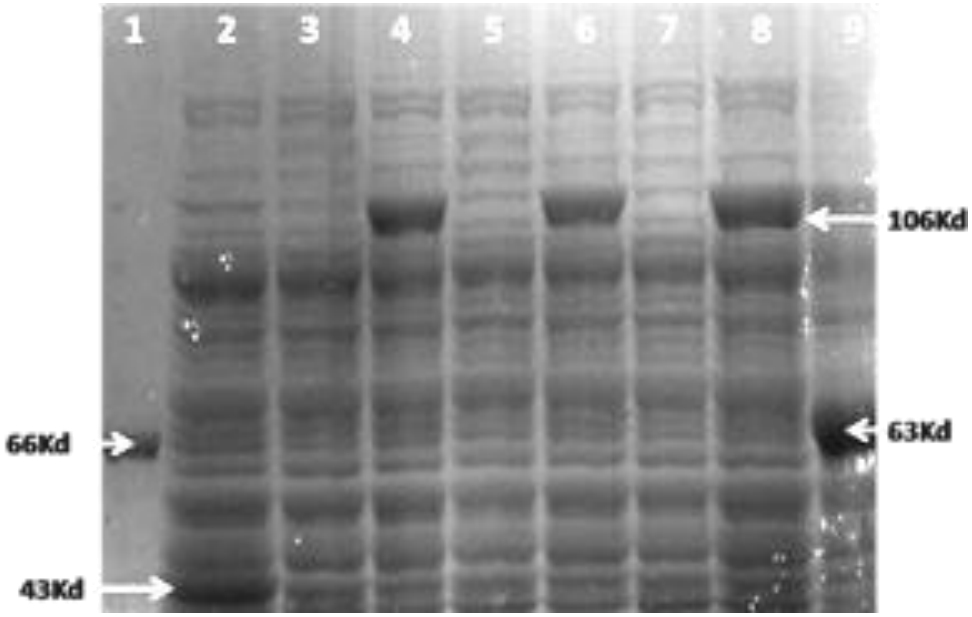

Figure 3. Protein profiles of *E. coli* BL21 expressing inulosucrase enzymes, resolved on 10% resolving gel by SDS-PAGE. Lane 1, BSA 66 kd; Lane 2, pMAL-p2 control; Lane 3, pMAL-p2ΔlacI^{Q}; Lane 4, pMAL-p2ΔlacI^{Q}-*inuJ* 106kd; Lane 5, pMAL-p2ΔlacI^{Q}; Lane 6, pMAL-p2ΔlacI^{Q}-*inuJ* 106kd; Lane 7, pMAL-p2ΔlacI^{Q}; Lane 8, pMAL-p2ΔlacI^{Q}-*inuJ* 106kd; Lane 9, pET-*inuJ* 63kd.

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

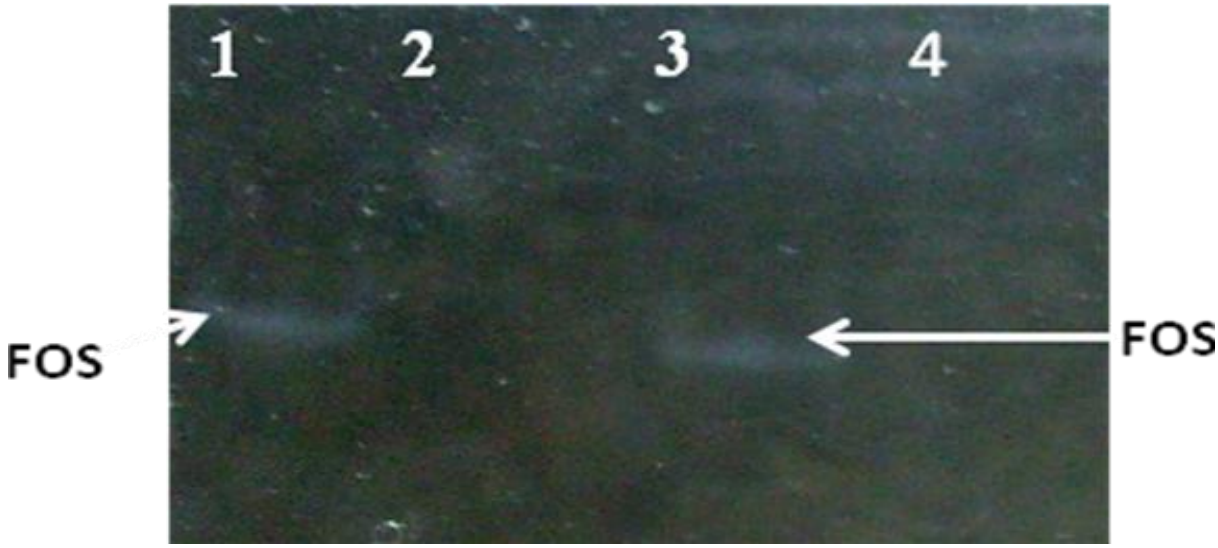

Figure 4. Native PAGE to show the activity of inulosucrase enzyme in supernatant of *E. coli* 16. White turbid band indicate FOS generation due to the activity of inulosucrase enzymes. Lane 1, pMALp2ΔlacI^{Q}-InuJ; Lane 2, pMALp2ΔlacI^{Q}; Lane 3, pMALp2ΔlacI^{Q}-InuJ; Lane 4, pMALp2ΔlacI^{Q}.

among others. Complex sugars like FOS on the other hand are beneficial and classified as functional food ingredient (Roberfroid, 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. coli*16 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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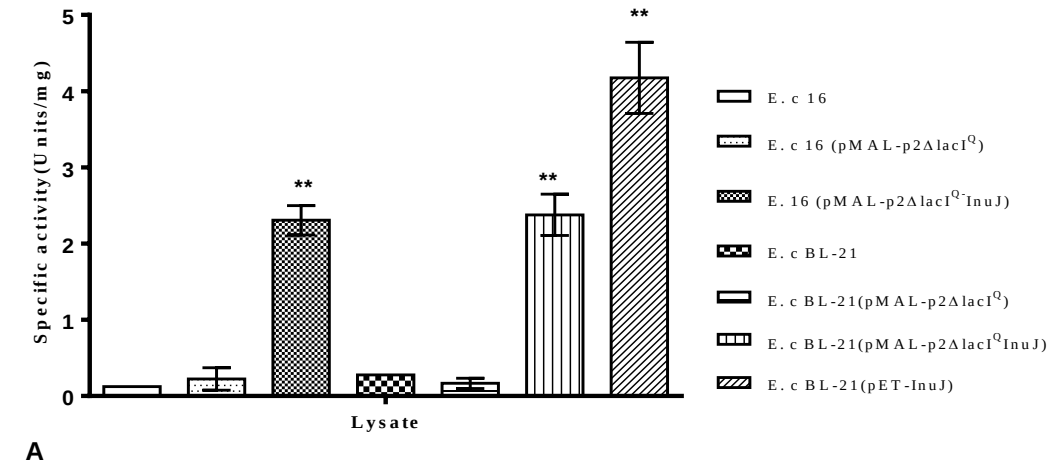

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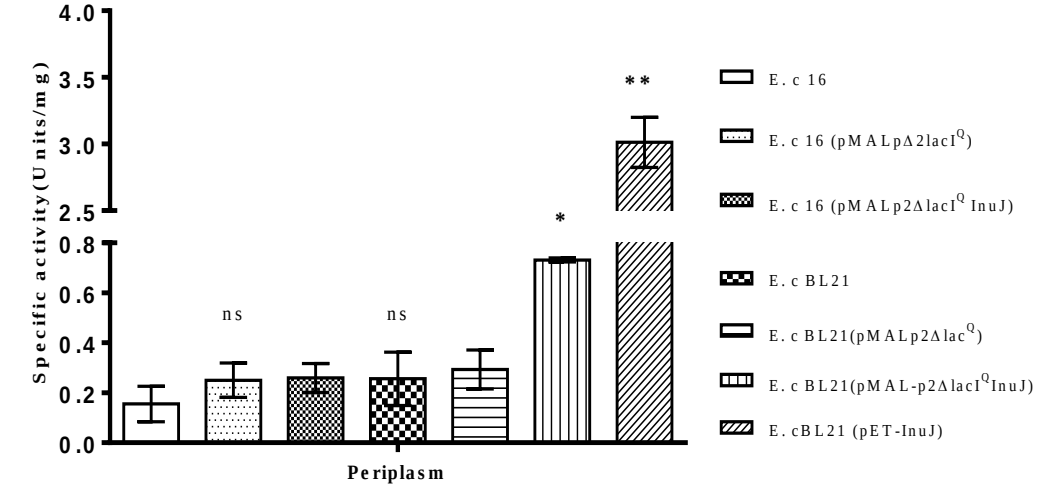

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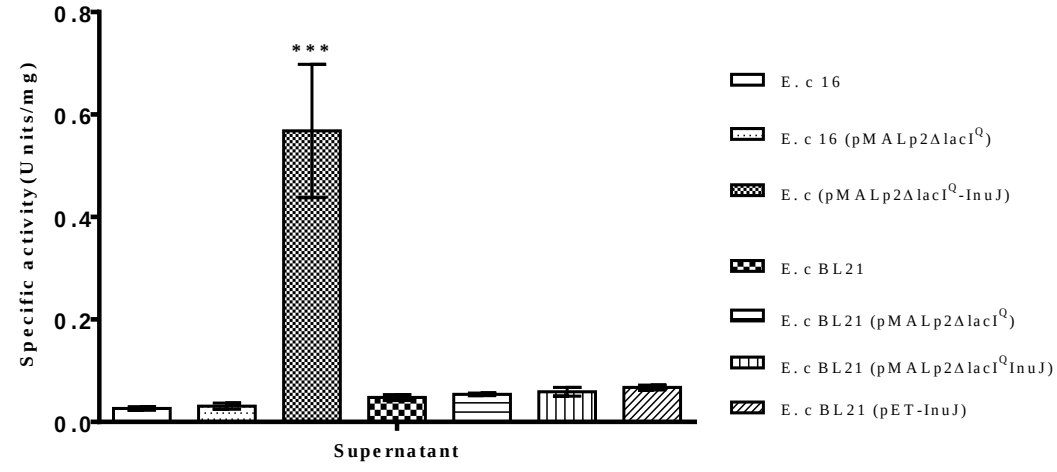

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Figure 5. Specific activity of inulosucrase enzyme in a, Lysate; b, periplasm and c, supernatant of *E. coli* 16 and BL-21 strain containing (pMAL-p2ΔlacIQ-InuJ). All values are expressed as mean ± SD (n=4-6) each group and analysis was done using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

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