Production, purification and physicochemical characterization of D-xylose/glucose isomerase from *Escherichia coli* strain BL21

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Abstract

Cell lysate of *Escherichia coli* strain BL21 showed significant D-glucose isomerase activity. The rate of glucose conversion was increased up to 40% when cells were induced with 1% D-xylose. *E. coli* BL21 xylose isomerase (ECXI-BL21) was purified to homogeneity, up to 1.9-fold with overall 10.88% enzyme yield by heat shock, salting out and electro-elution. The molecular mass of ECXI-BL21 was estimated as 43.9 kDa on SDS-PAGE. $pH_{opt.}$ and $T_{opt.}$ of the enzyme were calculated as 7.0 and 50 °C, respectively. Activation energy (E_a) of ECXI-BL21 was 45 kJ/mol. Enzyme was stable from 30 to 55 °C and at pH range 6.0–8.0. ECXI-BL21_(holo) was activated by 10 mM magnesium (35%), 0.5 mM cobalt (20%) and manganese (25%), and 0.5/10 mM Mn^{2+}/Mg^{2+} (50%) and Co^{2+}/Mg^{2+} (30%) as compared to ECXI-BL21_(apo). Catalytic affinity (K_m) of ECXI-BL21 for D-glucose was calculated as 0.82 mM, while maximum velocity (V_{max}) of the reaction $D\text{-glucose}_{(aldo)} \rightleftharpoons D\text{-fructose}_{(keto)}$ was 108 $\mu\text{mol}/\text{mg}/\text{min}$. D-fructose formed was identified on silica gel plate. This thermophilic enzyme, $T_m = 75$ °C, has great potential for high fructose syrup production used in food and soft drink industries.

Keywords *Escherichia coli* · Electro-elution · Kinetics · Thin-layer chromatography · D-glucose isomerase · Inducers

Introduction

Rapid depletion of fossil fuels diverted the attention of biotechnologist towards the development of efficient technology for bioconversion of renewable biomass into bio-fuel. Cellulose can be completely hydrolyzed to glucose which subsequently fermented to fuel ethanol. However, the utilization of all the components of hemicelluloses is a big challenge, as D-xylose which is one of the major sugars derived from biomass after glucose and comprised upon 40% of hemicelluloses, remained un-fermented, because the fermenting organisms, i.e., *Saccharomyces cerevisiae* can ferment hexoses very efficiently but not pentoses (Wang et al. 1980a). However, saprophytic bacteria flourished on rotting wood and decaying plant material used D-xylose_(aldo) $\rightleftharpoons$ D-xylulose_(keto) activity as energy source (Bhosale et al. 1996). Hence, this activity catalyzed by D-xylose isomerase (XI)

(D-xylose ketol isomerase; EC 5.3.1.5), can be utilized for the production of bio-ethanol from ligno-cellulosic biomass. D-xylulose, the isomerized product of D-xylose, has been fermented by conventional yeasts strains, i.e., *S. cerevisiae*, *Schizosaccharomyces pombe* and *Candida tropicalis*, very efficiently (Wang et al. 1980a, b; Chiang et al. 1981a, b; Schneider et al. 1981; Gong et al. 1981; Chan et al. 1989). XI also showed aldo $\rightleftharpoons$ keto conversion of D-glucose as well, hence also known as glucose isomerase (GI, EC 5.3.1.5), although the catalytic affinity of GI for hexoses is much lower than pentoses. D-glucose_(aldo) $\rightleftharpoons$ D-fructose_(keto) activity is extensively utilized in industry for high-fructose corn syrup (HFCS) manufacturing from corn starch. This process is comprised upon three successive enzymatic steps: liquefaction by α -amylase, saccharification by amyloglucosidase and isomerization of D-glucose to D-fructose by GI. HFCS has many advantages over sucrose in food industry, because it does not caused crystallization problem, has 10–20% low price (10–20%) due to 1.3 times higher sweetness power. Therefore, annual world-wide production of HFCS has been exceeded to 13 million tons (Rozanov et al. 2009; Staudigl et al. 2014).

Xylose isomerases (XIs) are intracellular, bacterial enzymes, classified as class I and II, based upon number

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of amino acids, they possessed in their protein sequences (Vangrýsperre et al. 1990). Class I enzymes are shorter (comprised upon ~390 amino acids) than class II (comprised upon ~440 amino acids) enzymes. Class II enzymes have additional 40–50 amino acids' insert at their N-terminus and are more varied in their source organisms from mesophiles (– 5–20 °C), thermophiles (15–45 °C) to hyperthermophiles (≥ 80 °C) as compared to class I which are more commonly mesophilic enzymes (Sriprapundh et al. 2000, 2003; Vieille and Zeikus 2001; Epting et al. 2005). XIs are tetrameric metallo-proteins, the catalytic site in each monomer is comprised upon one active and two metal-binding sites designated as M1 and M2 (Fig. 1). XIs required divalent metal ions chosen from Mn^{2+} , Co^{2+} and Mg^{2+} for their activity and stability (Collyer et al. 1990; Van Bastelaere et al. 1992; Bhosale et al. 1996) Xylose metabolism has been described from a wide array of microorganisms, most of which produced XIs only in the presence of D-xylose. *Escherichia coli* is a well-known Gram-negative bacterium, established in each laboratory. Hence, extensively used for homologous and heterologous protein production. *E. coli* is a fast-growing organism that utilized inexpensive growth medium and can metabolize both hexose and pentose sugars

as energy source. Hence, this system has been established on large scale and used for pharmaceutical and industrial proteins' production. A disadvantage of *E. coli* system is, accumulation of endo-toxins or lipopolysaccharide (LPS) as by product, which render unfit the protein produced for human consumption, because LPS are pyrogenic for human and other mammals. Therefore, the protein produced by this system must be purified subsequently for human use, to become endo-toxin free (Terpe 2006). This is the first report of production, purification and physicochemical characterization of xylose/glucose isomerase from *E. coli* BL21 (ECXI-BL21).

Materials and methods

Strain and chemicals

The cells of *Escherichia coli* BL21 (*F⁻ompThsdSB (rB⁻mB⁻) gal dcm(DE3)*) CodonPlus® (Cat. #69387-3) Novagen® were stored in 20% glycerol at ultra-low temperature (– 80 °C). All chemicals used of analytical grade and purchased from BDH (UK), E-Merck (Germany), Acros (Belgium), Fluka (Switzerland) and Sigma (USA).

Production of ECXI-BL21

Seed cultures were prepared in 100 ml flasks containing 10 ml of low-salt Luria Bertani (LB) broth (Tryptone 10 g, yeast extract 5 g, NaCl 5 g per liter). The flasks were incubated at 37 °C, pH 7.0, 150 rpm, overnight, after adding a single colony of *E. coli* cells which was previously grown on LB agar plates (1.5% bacteriological agar). Fifty milliliters of LB broth (pH 7.0) were sterilized at 121 °C, 15 lbs/in² pressures for 15 min in 250 ml flasks and cooled to room temperature. Seed cultures were diluted to 2% in to each flask (250 ml) individually and agitated (200 rpm) at 30–45 °C, pH 4–10 for 12–72 h. Meanwhile, when turbidity (O.D at 600 nm) was reached to 0.5–0.6, cells were induced with D-xylose, D-glucose, D-fructose, glycerol, maltose and lactose (filter (0.22 μ m) sterilized separately) by adding 1% of each carbon source in each flask separately. A control (without inducer) was also run parallel.

Purification of ECXI-BL21

Cells were harvested by centrifugation at $6000 \times g$, 4 °C for 10 min. Pellets were re-suspended in Tris–HCl buffer (100 mM, pH 7.5) and cell lysates were prepared by passing 30 short burst of sound waves at 4 °C, having an amplitude of 75% for 20 s followed by intervals of 40 s, using Hielsher Ultrasound Technology. Cell debris was removed by centrifugation for 15 min, at 4 °C (13,000 rpm). Supernatant was

Fig. 1 Monomer of ECXI, showing relative active site and either of the metal-binding sites residues in activated enzyme–substrate complex. ~50 extra amino-acid residues (blue) at N-terminus (not commonly present in class I enzymes) indicating that ECXI is class II enzyme (predicted by PatchDock v.β.1.3 using sequence of ECXI retrieved from NCBI (GenBank accession number E24377A) created by UCSF Chimera v.1.10.2 molecular graphic program)

lyophilized to about 50% reduction of the original volume. Then, it was heated for 15 min at 50 °C, 100 rpm and cooled on ice. The precipitated proteins were removed at 4 °C, by centrifugation for 15 min (12,000×g) and the supernatant (5 µl) was resolved on SDS-PAGE (12% acrylamide, w/v) along with pre-stained protein marker (Laemmli 1970). In supernatant, ammonium sulphate crystals were dissolved at 0 °C with constant stirring and precipitated fractions were collected (13,000 rpm, 4 °C) at 30–90% salt saturation individually. Fraction samples were re-suspended in Tris–HCl buffer (100 mM, pH 7.5) separately, sealed in cellulosic dialysis membrane, having a molecular weight cut-off 12,000–14,000. The membrane was previously treated with 1% acetic acid and basic EDTA solution containing 10^{-3} M EDTA and 1% Na_2CO_3 (Boyer 1993). Salt was removed by three replacements of same buffer then dialyzed sample (5 µl) was resolved (12% SDS-PAGE). This gel was used as reference for cutting the band from non-denatured gel. Native PAGE was prepared without wells (Fig. 2, step 1) in accordance to (Driska 1998; Branco et al. 2008; Li et al. 2012) and rest of the whole dialyzed sample was resolved on 10% acrylamide (w/v). The gel was stained with coomassie brilliant blue R-250 (0.25%) and de-stained with methanol (30%) and glacial acetic acid (10%). Bands were cut out with sharp scalpels and gel fragment containing band of interest was bounded in dialysis tube (Fig. 2, step 2 and 3). The tube was assembled in horizontal electrophoretic apparatus

filled with electrode-running buffer (pH 8.8) containing 250 mM glycine and 25 mM Tris–HCl. Electro-elution was performed at 100 V constant voltages until all the coomassie brilliant blue had been removed from the gel fragment (Fig. 2, step 4). The clear gel slice was removed from dialysis tube (Fig. 2, step 5) and the eluted protein was dialyzed at 4 °C against the three changes of Tris–HCl buffer (100 mM, pH 7.5) (Fig. 2, step 6). The purity of eluted protein was analyzed by SDS-PAGE (12% acrylamide, w/v). Protein contents of the samples at each step of purification were quantified against BSA (Bradford 1976).

Enzyme assays

A reaction mixture of 30 µl enzyme and 20 µl test solution containing 100 mM D-glucose, 0.0003 M MnCl_2 in Tris–HCl buffer (100 mM, pH 7.5) was incubated at 40 °C, for 10 min, using 96-well microplate. The plate was incubated on ice for 10 min, to stop the reaction. The amount of fructose produced was estimated in accordance to Schenk and Bisswanger (1998). One hundred and fifty micro-liters of 1:1 mixture (v/v, mixed freshly) of 0.05% resorcinol solution (in absolute ethanol) and 0.216 g/L ferric ammonium sulphate dodecahydrate solution in concentrated hydrochloric acid, were added. Plate was incubated at 80 °C for 45 min. The absorbance of red color developed was read by ELISA reader at 490 nm. One unit (U) of isomerase activity

Fig. 2 Purification process of ECXI-BL21. (1) 10% Native PAGE of ECXI-BL21 fraction obtained at 70% ammonium sulphate saturation, (2) Gel fragment containing ECXI-BL21, (3) ECXI slice sealed in dialysis membrane, (4) Electro-elution, (5) Gel slice without ECXI-BL21, (6) Dialysis, (7) SDS-PAGE

Fig. 3 Production of ECXI-BL21. Effect of time of incubation (a), effect of temperature (b), effect of pH (c), effect of inducers (d). Error bars show the standard error among parallel replicates ($n=3$)

Table 1 Purification of *E. coli* BL21 xylose isomerase

Purification steps	Total protein (mg)	Total activity (U/mg)	Specific activity (U/mg)	Overall yield (%)	Purification fold
Crude extract	525	10,333.5	19.68	100	1
Heat treatment	327.76	8097.6	24.7	78.36	1.25
70% $(\text{NH}_4)_2\text{SO}_4$	35	980	28	9.48	1.42
Electro-elution	30	1125	37.5	10.88	1.9

was “the amount of ECXI that produced 1 μmol fructose per ml, per min under specified conditions”. For kinetics constants (K_m and V_{max}), the concentration of D-glucose was varied (1–10 mM) in the reaction mixture and ECXI-BL21 activities were measured as mentioned above. Optimum pH was measured using different buffers (0.1 M), i.e., glycine–HCl (pH 2.0–3.0), sodium acetate (pH 4.0–5.0), sodium phosphate (pH 6.0–7.0), Tris–HCl (pH 8.0), glycine–NaOH (pH 9.0–10). The $\Delta pK_a/\Delta T$ was considered while calculating the results (Dawson et al. 1969). pH stability was estimated by incubating ECXI-BL21 at 50 °C, for 60 min under various pH (2–10) conditions individually then residual ECXI-BL21 activities were calculated at pH 7.0. Thermophilicity was calculated by performing the isomerase

reactions at various temperatures (30–90 °C) separately. $\ln V_{max}$ at each temperature treatment was plotted as Arrhenius plot (linear between 30–50 °C) and activation energy (E_a) was calculated by applying the equation $E_a = -\text{slope} \times R$ where, R = gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$). Thermostability studies were performed by incubating ECXI-BL21 in the absence of substrate at each temperature 30–90 °C individually for 60 min. Residual ECXI-BL21 activities were calculated at 50 °C. ECXI-BL21 was incubated overnight, in Tris–HCl buffer (100 mM, pH 7.5) containing 10 mM EDTA at 4 °C. It was then dialyzed twice against the same buffer containing 2 mM EDTA (at 4 °C) and finally dialyzed twice without EDTA (at 4 °C). The apo-ECXI-BL21 (metal free) thus obtained was equilibrated individually with 0.3, 0.5, 5,

Fig. 4 SDS-PAGE of ECXI-BL21. **a** Lane 1: after heat treatment, Lane 2 and 3: before heat treatment, Lane 4: ACT GeneTM (P/N: ACT-IDWW24) pre-stained protein marker (11–245 kDa). **b** Lane 1: ACT GeneTM (P/N: ACT-IDWW24) pre-stained protein marker, Lane 2: after electro-elution

and 10 mM chloride salts of Mn^{2+} , Co^{2+} and Mg^{2+} and in combination (0.5/10 mM) Mn^{2+}/Mg^{2+} and Co^{2+}/Mg^{2+} at 30 °C, for 30 min. The percentage increase or decrease in activities of holo-ECXI-BL21 (metal equilibrated enzyme) was calculated at 50 °C, relative to apo-ECXI-BL21. The final product of glucose conversion achieved at optimized reaction conditions was identified by thin-layer chromatography (TLC). All assays were performed in parallel triplicates.

TLC analysis

Silica gel (Kiesel gel 60 F254; Merck) plate was developed by double-development technique using ascending method as described previously (Fatima et al. 2016). D-glucose and D-fructose each of 10 mg·ml⁻¹ were spotted as standards. Air-dried developed, plate was sprayed with 5% conc. H_2SO_4 (v/v, in methanol) and incubated at 150 °C, for 10 min, in hot air oven. Resultant spots were identified by retention factor (Rf) and compared with standards (Walker et al. 1965).

Statistical analysis

Graph Pad Prism 6 software was used to calculate means and standard errors among the treatments ($n = 3$).

Results and discussion

Production of ECXI-BL21

The maximum D-glucose isomerase activity (146.67 ± 1.2 U/ml/min) was achieved at 37 °C, pH 7.0 after 48 h (Fig. 3a–c) which was independent of any carbon source induction. However, the enzyme activity was increased when *E. coli* BL21 cells were induced with different carbon sources (Fig. 3d). As compared to control (146.67 ± 1.3 U/ml/min), a maximum 41.09% increase in enzyme activity (206.67 ± 0.5 U/ml/min) was observed when fermentation medium was supplemented with 1% D-xylose. Similarly, *Bacillus megaterium* cells showed maximum D-glucose isomerase activity at 37 °C, pH 7.0 when induced with 1% D-xylose (Nguyen and Tran 2018). The first report on xylose transport in *E. coli* X289 revealed that xylose permease system is specifically induced by D-xylose (David and Weismeyer 1970). D-xylose transport system in *E. coli* is linked with a protein, which only take up D-xylose as the pH changed to alkaline conditions (Lam et al. 1980). However, *E. coli* BL21 cells produced highest enzyme titer at pH 7.0. Later on, a periplasmic xylose-binding protein was purified from *E. coli* K-12 (Ahlem et al. 1982). This protein is released

Fig. 5 Effect of reaction conditions on ECXI-BL21 activity and stability. Effect of pH and pH stability (a), effect of temperature and thermostability (b), Arrhenius plot ($R^2=0.941$) (c). Error bars show the standard error among parallel replicates ($n=3$)

only by osmotic shock and is an integral part of high-affinity xylose transport system reported for closely related enteric bacterium *Salmonella typhimurium* LT2. However, it is very interesting that this binding protein was produced when *S. typhimurium* LT2 was grown on medium containing xylose as well as glycerol (Shamannat and Sanderson 1979). Previously, among all the carbon sources used,

maximum D-glucose isomerase activity ($D\text{-glucose}_{(aldo)} \rightleftharpoons D\text{-fructose}_{(keto)}$) was achieved when *E. intermedia* cells were induced by 2% D-glucose. However, cells of *E. intermedia* not showed any D-xylose isomerase activity ($D\text{-xylose}_{(aldo)} \rightleftharpoons D\text{-xylulose}_{(keto)}$) activity when induced with carbon sources other than D-xylose (Natake and Yoshimura 1964). It might due to repressive effects of other sugars by which they were not transported into *E. intermedia* cells (Ammar et al. 2018).

Purification of ECXI-BL21

ECXI-BL21 purified to homogeneity, up to 1.9-fold by heat treatment, salting out and electro-elution. Overall, 10.88% yield of enzyme was achieved (Table 1). Heat treatment of ECXI-BL21 was very efficient, as more than 50% contaminants (other proteins) were removed at this step (Fig. 4a). Previously, ECXI-K12 was purified to homogeneity with an overall yield of 18% (Schellenberg et al. 1984). The purified ECXI-BL21 was resolved on 12% SDS-PAGE. The molecular mass (M_r) of ECXI-BL21 monomer was calculated as 43.9 kDa using calibration curve (Fig. 4b). Similarly, a thermophilic class I enzyme from *Thermobifida fusca* MBL 10,003 showed $M_r=43.9$ kDa (Kasumi et al. 2012). Among class I, mesophilic enzyme from *Streptomyces* sp.CH7 has $M_r=43.6$ kDa (Chanitnun and Pinphanichakarn 2012), while hyperthermophilic, class II enzyme from *Thermotoga maritima* MSB8 has $M_r=45$ kDa (Brown et al. 1993). It was previously reported that a ~1.3 kb (1320 bp) gene (*xyl-A*) fragment of *E. coli* K12 encoding for XI of 440 amino acid residues long has a $M_r=44$ kDa (Schellenberg et al. 1984). Hence, ECXIs are class II enzymes.

Physicochemical characterization of ECXI-BL21

The optimum pH of ECXI-BL21 was calculated as 7.0 and by further increase in pH to 8.0, it showed 15% less activity than optimal (Fig. 5a). Similarly, the maximum catalytic activity of ECXI-K12 was calculated as 6.8 (Rozanov et al. 2009). This value is very close to our finding. The neutral pH optima of ECXIs is a good sign as industries utilizing this enzyme demanded slightly acidic to neutral pH optima of the enzyme for HFCS production (Bhosale et al. 1996). Class II, hyperthermophilic enzyme from *T. naphthophila* (Fatima et al. 2016), thermophilic enzymes from *Caldanaerobacter subterraneus* sub sp. *yonseiensis* (Kim et al. 2010) and *B. thuringiensis* (El-shora et al. 2016) and among class I, enzymes from *Streptomyces* sp. CH7 (Chanitnun and Pinphanichakarn 2012) and *S. albaduncus* (Yassien et al. 2013) also showed optimum activity at pH 7.0. ECXI-BL21 was completely unfolded under highly acidic conditions as the

Fig. 6 Effect of divalent metal ions on ECXI-BL21 activity. Effect of Mg^{2+} (a), effect of Co^{2+} (b), effect of Mn^{2+} (c), effect of Mg^{2+} with Co^{2+} and Mn^{2+} (d). Error bars show the standard error among parallel replicates ($n = 3$)

enzyme did not show isomerase activity at pH 2.0 and 3.0. Catalytic activity of ECXI-BL21 was reduced to 30 and 40% at pH 4.0 and 5.0, respectively. ECXI-BL21 was stable at pH range 6.0–8.0 when incubated at 50 °C, in the absence of substrate (Fig. 5a). ECXI-BL21 activity was reduced to 14–15% when it was incubated at pH 9, for 60 min. The hyperthermophilic, class II enzymes from *T. neapolitana* DSM 5068 and *T. naphthophila* RKU-10 retained 100 and more than 80% of their catalytic activities at pH range 6.8–7.3 and 6.0–8.0, when incubated for 30 min at 90 and 80 °C, respectively (Vieille et al. 1995; Fatima et al. 2016).

Optimal temperature of ECXI-BL21 was determined as 50 °C (Fig. 5b). Similarly, enzyme from *B. thuringiensis* has optimum temperature 50 °C which is also a thermophilic, class II enzyme (El-shora et al. 2016). ECXI-BL21 was stable from 30 to 55 °C. By further increase in temperature, enzyme undergoes fast denaturation ($T_m = 75$ °C), as about 12% reduction in its catalytic activity was noted at 60 °C (only by 5 °C increase in temperature) and it was 80% reduced at 90 °C (Fig. 6b). Similarly, ECXI-K12 commenced for denaturation at 45 °C and its catalytic activity was 100% lost at 65 °C, after 60 min of incubation. However, it was optimally active at 45 °C (Rozanov et al. 2009). Hence, ECXI-BL21 is more active at higher temperature than ECXI-K12. The activation energy of ECXI-BL21 for

D-glucose_(aldo) $\rightleftharpoons$ D-fructose_(keto) conversion was calculated as 45 kJ/mol (Fig. 5c).

The catalytic activity of ECXI-BL21_(holo) was increased to 35% as compared to ECXI-BL21_(apo), when it was equilibrated with 10 mM Mg^{2+} (Fig. 6a) and it was reduced by 10 and 15% when Mg^{2+} was replaced with 0.5 mM Co^{2+} (Fig. 6b) and Mn^{2+} (Fig. 6c), respectively. However, the catalytic activity of ECXI-BL21_(apo) was increased maximum to 15% when it was equilibrated with 0.5 mM Mn^{2+} and 10 mM Mg^{2+} simultaneously (Fig. 6d) as compared to Mg^{2+} alone. Maximum 50% increase in enzyme activity of ECXI-BL21_(holo) was achieved by Mn^{2+}/Mg^{2+} combination as compared to ECXI-BL21_(apo). Similarly, 30 mM Mg^{2+} enhanced the catalytic activity of ECXI-K12 and it was further maximized up to 15–20% when 20 mM Mg^{2+} along with 10 mM Co^{2+} was simultaneously present in the reaction mixture (Rozanov et al. 2009). Class II, thermophilic XIs from *C. subterraneus* sub sp. *yonseiensis* (CSXI) (Kim et al. 2010), *Fervidobacterium gondwanense* (FGXI) (Kluskens et al. 2010), *Anoxybacillus gonensis* G2T (AGXI) (Yanmis et al. 2014) and hyperthermophilic XI from *T. maritime* MSB8 (TMXI) (Brown et al. 1993) required Co^{2+}/Mg^{2+} at 1/10 mM concentration for their optimal performance. It is reported that the addition of Co^{2+} should be avoided due to their health and environmental hazards, because of human

Fig. 7 Separation of reducing sugars on kiesel gel plate. **a** D-glucose, **b** D-glucose after isomerization catalyzed by ECXI-BL21, **c** D-glucose + ECXI-BL21 before isomerization, **d** D-fructose

consumption of HFCS and industrial effluent, respectively (Bhosale et al. 1996). ECXI-BL21 is superior to ECXI-K12, CSXI, FGXI, AGXI and TMXI as it does not require Co^{2+} for its optimal performance. On silica gel plate, ECXI-BL21 reaction product, produced under above-mentioned optimized conditions appeared as black spots (Fig. 7). The retention factor of D-glucose (Lane 1) and D-fructose (Lane 4) was calculated as 0.62 and 0.65, respectively. A considerable amount of D-glucose was isomerized to D-fructose as depicted by Fig. 7 (Lane 2). There is scarcity of the literature for the comparison of ECXI-BL21 with same enzyme from other strains of *E. coli*.

Activity data of ECXI-BL21, at different substrate concentrations, were plotted as non-linear fit of Michaelis–Menten equation (Fig. 8a) and then transformed as double reciprocal plot (Fig. 8b) (Lineweaver and Burk 1934), using Graph Pad Prism 6. The K_m and $V_{\max}$ values of ECXI-BL21_(holo) at 50 °C, pH 7.0 for glucose_(aldo) to fructose_(keto) conversion were calculated as 0.82 mM and 108 $\mu\text{mol}/\text{mg}/$

Fig. 8 Kinetics of ECXI-BL21. **a** Michaelis–Menten plot, **b** Lineweaver–Burk plot ($R^2=0.996$)

min, respectively, when it was equilibrated with 0.5 mM Mn^{2+} and 10 mM Mg^{2+} . However, Rozanov et al. (2009) reported $V_{\max}=0.06 \mu\text{mol}.\text{mg}^{-1}.\text{min}^{-1}$ and $K_m=0.22 \text{ l/mol}$ at 30 °C; $V_{\max}=0.8 \mu\text{mol}.\text{mg}^{-1}.\text{min}^{-1}$ and $K_m=0.5 \text{ l/mol}$ at 45 °C of ECXI-K12 for D-glucose_(aldo) $\rightleftharpoons$ D-fructose_(keto) conversion at 30 mM Mg^{2+} , pH 6.8. Again, ECXI-BL21 is superior to ECXI-K12 as it showed greater catalytic affinity and maximum velocity at higher temperature than K12enzyme, because high temperature shifts equilibrium of the reaction in forward direction (towards fructose). Currently, immobilized XIs used in industries, under trade name Spezyme IGI, Optisweet 22, Gensweet SGI, AGIS-600, Sweetase, Sweetzyme T and Maxazyme from *S. rubiginosus*, *S. rubiginosus*, *S. murinus*, *S. griseofuseus*, *S. phaeochromogenes*, *S. murinus* and *Actinoplanes missouriensis* manufactured by Finnsugar, Finland; Miles Kali-Chemie, Germany; Genencor, USA; Godo-Shusei, Japan; Nagase, Japan; Novo-Nordisk, Denmark and Gist-Bocades, Netherlands, respectively (Jensen and Rugh 1987), are all mesophilic, class I enzymes. For D-glucose_(aldo) $\rightleftharpoons$ D-fructose_(keto) conversion the K_m and $V_{\max}$ of XIs from *S. griseofuseus*, *S. olivochromogenes*, *Streptomyces* sp CH7 and *A. missouriensis* were

reported as 220, 250, 258 and 230, 290 mM and 17.6, 5.33, 32.42 and 33.9 U/mg, respectively (Suekane et al. 1978; Kasumi et al. 1982; Smith et al. 1991; Jenkins et al. 1992; Van Bastelaere et al. 1995; Chantitnun and Pinphanichakarn 2012). These results showed that ECXI-BL21 showed greater catalytic affinity for D-glucose than all commercially available XIs currently used in industries.

Conclusion

In conclusion, ECXI-BL21 can be produced from well-known already-established system of *E. coli* BL21(DE3) Codon Plus. ECXI-BL21 was purified very efficiently by electro-elution as compared to column chromatography which is expensive and time-consuming technique. ECXI-BL21 showed highest catalytic affinity and maximum velocity, required lowest concentration of Mn^{2+} and not required Co^{2+} at all, as compared to other XIs reported to date. The enzyme is thermophilic with neutral pH optima as demanded by industries for HFCS production. Also, it has broad range pH and temperature stability. Hence, ECXI-BL21 could be a best candidate for fructose syrup production.

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Author contribution MMJ conceived and supervised the study. BF designed and performed the experiments, analysed the data and wrote the manuscript. Both authors contributed to the discussion and revision of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest related to this article.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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