ADDENDUM

Expression of thermostable \( \beta \)-xylosidase in *Escherichia coli* for use in saccharification of plant biomass

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**ABSTRACT**

The present work is aimed to evaluate the saccharification potential of a thermostable \( \beta \)-xylosidase cloned from *Bacillus licheniformis* into *Escherichia coli* for production of bioethanol from plant biomass. Recombinant \( \beta \)-xylosidase enzyme possesses the ability of bioconversion of plant biomass like wheat straw, rice straw and sugarcane bagass. By using this approach, plant biomass that mainly constitute cellulose can be converted to reducing sugars that could then be easily converted to bioethanol by simple fermentation process. The production of bioethanol will help to overcome energy requirements due to depleting fossil fuels and will also help to protect environment by reducing greenhouse gas emission. In the end, future directions are briefly mentioned that can be utilized to reduce the cost and increase the yield of biofuels.

\( \beta \)-xylosidases are important industrial enzymes that are required for maximum hydrolysis of hemicellulose, xylan, in synergistic effect with xylanases. \( \beta \)-xylosidases play an important role in breaking down of xylo-oligosaccharides, a component of xylan, to xylose. Recently, we have cloned a \( \beta \)-xylosidase enzyme from *Bacillus licheniformis* and expressed successfully in *E. coli* for use in saccharification of plant biomass. In this addendum, expression of cloned \( \beta \)-xylosidase enzyme in *E. coli* and its saccharification potential for plant biomass are discussed.

Fossil oil is predominantly used as fuel for transportation and in industry. Due to its increased usage, it has been predicted that fossil fuel reserves may come to a point of near depletion around 2050\(^1\). Further constant use of fossil fuel, elevated the CO\(_2\) emission from 355 ppm in 1991 to 391 ppm in 2011\(^2\). However, greenhouse gas emission can be dramatically reduced by use of bioethanol\(^3\). It is proposed that replacement of fossil fuel with bioethanol can be achieved before 2050\(^\). The total annual primary production of biomass is over 100 billion tonnes of carbon per year and the energy reserve per metric ton of biomass is between 1.5 E3–3 E3 kilowatt hours that are sufficient to cater the needs of the world energy requirements\(^6\). It is however, essential to understand the composition of plant biomass in order to convert it into final product of bioethanol. Plant biomass consists of cellulose, hemicellulose and lignin. The most common constituent of the plant biomass (lignocellulose) is cellulose (40–60%) consists of linear chains of glucose monomers linked in 1–4 \( \beta \)-configurations (with high molecular weight of 100,000) and arranged into microfibrils\(^7\). Hemicellulose (20–40%) abundantly xylans and xyloglucans (with molecular weight of 30,000) is linked via hydrogen bonds to microfibrils\(^8\). Lignin (10–25%) is formed by the polymerization product of phenyl-propanes with high molecular weight\(^9\). \( \beta \)-xylosidase enzyme hydrolyses 1–4, \( \beta \)-D-xylans to remove successive D-xylose residues from non-reducing termini.

Lignocellulose (40–60%) is one of the main components of plant source for bioethanol production as an alternative energy source, as it is not in direct competition with food sources\(^10\). Therefore, it is a potential candidate for the generation of sugar (xylose) that in turn can be utilized for the production of bioethanol. Complete hydrolysis of xylan into useful end product i.e. xylose, requires a synergetic action of xylanolytic enzymes (endoxylanase and \( \beta \)-glucosidase)\(^11\). Endoxylanases hydrolyze the xylan backbone randomly and produce xylooligosaccharides which are

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converted into fermentable sugar, xylose, by the action of β-xylosidases. So for the bioconversion of lignocellulosic biomass into xylose, there is a significant value of highly specific β-xylosidases.

Recently we had cloned a xylanase gene from *Bacillus licheniformis*, expressed in *E. coli* BL21 (DE3) and checked its potential for the hydrolysis of different types of plant biomass. In order to obtain the maximum sugar released from plant biomass, effect of action of both xylanase and β-xylosidase was studied.

We have cloned a β-xylosidase (xynB) gene from *Bacillus licheniformis* and expressed the cloned gene in *E. coli* strain BL21 (DE3) using expression vector pET 21a(+). Finally saccharification potential of the cloned β-xylosidase gene for plant biomass was also determined. xynB gene was amplified by using specific pair of primers which were designed against nucleotide sequence (Accession No. Q65MB6) retrieved from NCBI database, ligated into pET 21a(+) expression vector after double restriction with NdeI and HindIII using T4 DNA ligase and transformed in freshly prepared competent cells of *E. coli* BL21 (DE3). Positive clones were screened by restriction analysis of recombinant plasmid.

Expression of recombinant xynB gene was analyzed by SDS-PAGE (Fig. 1). Different types of controls: cell lysate of wild *E. coli* BL21 (DE3), cell lysate of *E. coli* BL21 (DE3) containing only pET 21a(+) vector without any insert and cell lysate of recombinant *E. coli* BL21 (DE3) containing non-induced pET 21a(+) with gene of insert were run in parallel in order to investigate the expression of cloned xynB gene (Fig. 1). The apparent bands of 52 kDa was observed in cell lysate of recombinant *E. coli* BL21 (DE3) induced with IPTG while no band was observed in all controls which showed successful expression of cloned β-xylosidase gene. Sumarish et al. also expressed a β-xylosidase gene from *Bacillus megatarium* into *E. coli* using vector pHIS1525 showing 58 kDa size of the enzyme while Banka et al. expressed a β-xylosidase gene from *Bacillus subtilis* into *E. coli* using pFLAG-CTS vector and demonstrated the size of the enzyme around 60 kDa.

Further, recombinant β-xylosidase enzyme was analyzed for saccharification potential for different types of pretreated plant biomass i.e., rice straw, wheat straw and sugarcane bagass, as shown in Fig. 2. Different parameters including temperature, pH, time of incubation, enzyme concentration and substrate concentration were studied to obtain highest percentage of saccharification. It was observed that maximum saccharification (16%) of biomass was achieved at 50°C, pH 7.0 after 6 hours of incubation when 30 units of enzyme and 8% substrate (wheat straw) concentration was used. Saccharification of 5.5%, 6.8%, 8.2%, 6.4% and 6.4% was obtained when β-xylosidase was incubated along with sugarcane bagass at 30°C, 40°C, 50%, 60°C and 70°C respectively. In contrary, less saccharification percentage was achieved for rice straw and wheat straw; however, maximum biomass was converted into sugar at 50°C for all three biomass used (Fig. 2A). Characterization of the cloned β-xylosidase is not yet performed but it is obvious from results obtained from saccharification of biomass that β-xylosidase is thermostable and can tolerate high temperature for longer period of time. Mahamud and Gomes (2012) exhibited maximum hydrolysis (37.2%) of alkali-treated substrates at 50°C. Similarly, at different pH values of 5, 6, 7, 8 and 9, calculated saccharification percent was 4.5%, 5.3%, 6.4%, 5.3% and 3.9%, respectively with rice straw, 5.4%, 6.0% 7.3%, 6.3% and 5.3%, respectively with wheat straw and 5.9%, 7.5%, 8.2%, 6.5% and 4.8% respectively with sugarcane bagass. It is obvious from above results that maximum saccharification efficiency is achieved at pH 7.0 (Fig. 2B). It is well established that pH has a significant effect on the hydrolytic behavior of cellulases. The process of hydrolysis occurs only after formation of enzyme-substrate complex, and in this case the
adsorption and hydrolysis occurs simultaneously at around pH 7.0. It has been reported previously that pH 7.0 is optimum for saccharification of sugarcane bagass and other agro-wastes by using cellulase enzymes.\textsuperscript{19} Similar results were reported from cellulases of \textit{Bacillus subtilis}.\textsuperscript{20} However, some reports show that optimum saccharification occurred at pH 6.0.\textsuperscript{21,22} As far as time of incubation is concerned, maximum reducing sugars were released after 6 hours of enzyme-substrate incubation for all substrates (Fig. 2C). In one study, maximum saccharification (5.12\% and 7.31\%) from endoglucanase from \textit{Clostridium thermocellum} was achieved against wheat straw and bagass after 2 hours of incubation.\textsuperscript{23} Another study reported 4–12 hours of predicted time of incubation by using response surface methodology using cellulases enzymes.\textsuperscript{24} Similarly, enzyme concentration considerably effect the hydrolytic potential of \textit{\beta}-xylosidase and maximum saccharification percentage (13.5\%) was obtained after addition of 30 units of recombinant \textit{\beta}-xylosidase in reaction mixture containing sugarcane bagass while 10\% and 10.5\% saccharification was observed with both rice straw and wheat straw when same amount of enzyme was used (Fig. 2D).

Maximum reducing sugars were released from biomass after incubation with \textit{\beta}-xylosidase when 8\% concentration of substrates was used and saccharification

Figure 2. Optimization of saccharification potential of recombinant \textit{\beta}-xylosidase on rice straw, wheat straw and sugarcane bagass. (A) Effect of incubation temperature (B) Effect of pH (C) Effect of time of incubation (D) Effect of enzyme concentration (E) Effect of substrate concentration.
calculated in terms of percentage was 12.2%, 12.0% and 16.0% for rice straw, wheat straw and sugarcane bagasse respectively (Fig. 2E). Stenberg et al. (2000) demonstrated that highest yield of glucose and man- nose present in the original wood was obtained at 5% substrate concentration.25

To further enhance saccharification potential of recombinant β-xylosidase, purification and characterization of the enzyme will be performed and could be used more efficiently for saccharification of plant biomass which in turn will be used for bioethanol production.

Biofuel production and its replacement with fossil fuel is one of the biggest challenges for scientists to secure the future of our planet in terms of growing energy requirements and depletion of fossil fuel reserves at rapid pace. The cost is the most important factor in the production and commercialization of biofuel. To achieve this target, attention must be given to three main areas that include selection of biomass, pretreatment of cellulose biomass, enzymatic action on pretreated biomass, fermentation and product separation.

The cost and yield of biomass is influenced by various factors like soil fertility, genetics and location. Many genetic engineering techniques are being applied to boost the yield of specific plant like popular, switchgrass, Miscanthus and forage sorghum. Further genetic modifications in plants can be performed to increase the growth which include cell cycle mechanism, hormone metabolism, genes involved in photosynthesis, lignin alterations etc.

Pretreatment involves oxidizing agents, alkalis, acids, organic solvents and ionic liquids that degrade lignin, hemicellulose and cellulose from lignocellulosic biomass. Alternatively, environmentally friendly biological treatment involves the use of cellulolytic and hemicellulolytic organisms or peroxidases and laccases enzymes in pretreatment of biomass for delignification of lignocellulosic biomass. Further, mechanical pretreatment of lignocellulosic biomass is also being used for biofuel production. A major bottleneck in the pretreatment technology is the presence of lignin which is a major inhibitor of hydrolysis of cellulose and hemicellulose. No pre-treatment process provides 100 % conversion of biomass into fermentable sugars and is not performed at ideal conditions but it should be able to break the cell wall and transport lignin to the surface, produce few degradation products, use less energy, easily scaled up, carry out at less temperature and pressure and use less toxic chemicals.

To hydrolyze the pretreated lignocellulosic biomass, various hydrolytic enzymes, such as cellulases, hemicellulases and accessory enzymes are being used to attack hemicellulose debranching, phenolic acid esters and ligninases for lignin degradation/modification are required for complete deconstruction of the various components of lignocellulosic biomass. In this regard, cellulases and other hydrolytic enzymes are being used individually or in cocktail formation to reduce the intractable carbohydrate portion of pretreated lignocellulosic biomass. A major problem in the enzymatic treatment of biomass is the quick deactivation of cellulases and other enzymes. This problem can be eradicated by the immobilization of enzymes on suitable support. In addition, to reduce the processing cost, these enzymes should be recycled.

To convert sugars (glucose and xylose) into biofuels, microbial fermentation is performed which involves yeast, fungus and bacteria. Fermentation of glucose is generally performed to convert into biofuel as these microbes cannot convert xylose into ethanol. Genetic modifications can be executed in microbes to make them consume xylose for the production of biofuel. Recently, our laboratory has isolated a new strain of Wickheramomyces anomalus that has an ability to convert both glucose and xylose simultaneously into biofuel. Such organisms can be helpful to increase the yield of biofuel.

Distillation is generally used for separation of alcohol and water. Upto 95% pure ethanol can be achieved by distillation that require large amount of energy. However, further methods are required like discovery of molecular sieves or additives to obtain pure ethanol. A new approach like microbubble distillation technology is recently suggested that require less energy. Additionally, future biofuels that remain insoluble in water can be found that can easily be separated without distillation process.

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