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8.
Enzyme-coupled procedure for xylose conversion into biotechnologically important intermediates

Georgiana Petrareanu, Mihaela Balasu, Stefan Szedlacsek*

Institute of Biochemistry, Bucharest, Romania

The great majority of the actual research studies dedicated to finding new ways to valuate (ligno)cellulosic residues are directed to fermentative procedures. Less attention has been paid so far to the discovery of processes leading to high-value compounds. To overcome this shortcoming, we suggest a coupled enzymatic reaction procedure which can convert xylose – one of the most important pentose in lignocellulosic wastes – to high value biosynthetic intermediates. Specifically, xylose is initially isomerized to xylulose by xylose isomerase and then xylulose is phosphorylated to xylulose 5-phosphate by xylulose kinase. Finally, xylulose 5-phosphate is converted by D-xylulose 5-phosphate phosphoketolase to acetyl phosphate and glyceraldehyde 5-phosphate, both important intermediates for biosynthetic technologies. While the first two enzymes have been cloned and are well documented, cloning of phosphoketolase has been relatively recently reported (Meile et al., 2001). Therefore, we cloned the hypothetical phosphoketolase genes from four different organisms: *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Synechocystis* sp. and *Pseudomonas aeruginosa.* Each gene was subsequently inserted in a prokaryotic vector, then expression was optimized and expressed proteins were purified. Enzyme kinetic characterization of purified phosphoketolases necessitated setting up of a small scale procedure for preparation of xylulose 5-phosphate substrate (commercially not available). Thus, enzyme stability data, optimal reaction conditions and enzyme kinetic parameters were used in a comparative study aimed to find the optimal phosphoketolase for our purpose. Finally, all three enzymes of the coupled reaction were immobilized on inert supports in three individual columns, at laboratory scale. Preliminary experiments performed on the serially connected columns evidenced the successful conversion of xylose to acetyl phosphate and glyceraldehyde 5-phosphate.

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9.
Construction of amylolytic nuclear petite *Saccharomyces cerevisiae* strains for bioethanol production by SSF

Doga Arslan[1], Ozlem Ates[2,*], Ebru Toksoy Öner[1], Stephen G. Oliver[3], Betül Kirdar[4]

[1] *Department of Chemical Engineering, Marmara University Goztepe 34722, Istanbul, Turkey*
[2] *Department of Chemical Engineering, Yeditepe University Kayisdagi 34755, Istanbul, Turkey*
[3] *School of Biological Sciences, University of Manchester, Manchester, United Kingdom*
[4] *Department of Chemical Engineering, Boğaziçi University Bebek 34342, Istanbul, Turkey*

The energy intensive high temperature cooking process followed by enzymatic hydrolysis of starch to fermentable sugars in the current bioethanol production process warrants novel and innovative new technology for reducing the total manufacturing cost. The aim of the present study was to develop a novel noncooking fermentation system utilizing amylolytic microorganisms that are capable of hydrolyzing starch into fermentable sugars by enabling depolymerization of starch to glucose in a simultaneous saccharification and fermentation (SSF) process for bioethanol production. Respiratory-deficient nuclear petite *Saccharomyces cerevisiae* strains hold a great potential for the commercial production of bioethanol. Therefore, for the construction of the amylolytic yeast strains, the nuclear petite *S. cerevisiae* FY23Δpet191 strain was used as host. This 100% respiratory-deficient strain was generated using polymerase-chain-reaction (PCR)-mediated disruption of the PET191 gene which encodes a protein required for the assembly of the polypeptide subunits that constitute the active cytochrome c oxidase holoenzyme (Hutter and Oliver, 1998). The constructed yeast strains were found to excrete a bifunctional fusion protein that contains both the *Bacillus subtilis* alpha-amylase and the *Aspergillus awamori* glucoamylase activities (Toksoy Öner et al., 2005). The fermentation performances of these strains pointed out the fact that in order to use these strains on industrial scale, both the copy number and stability of the genes encoding the amylolytic enzymes had to be improved (Toksoy Öner, 2006). In the light of this, a cloning strategy was developed and used in order to integrate multiple copies of the fusion protein gene into the ribosomal DNA (rDNA) locus of *Saccharomyces cerevisiae* that consists of 100–200 tandem copies of a 9.1 kb repeat on the right arm of chromosome XII. 2.7 kb long fragment containing the gene for 25S rRNA was amplified by PCR and cloned into pJET1 cloning vector to construct pJRD. In order to construct the PJBG plasmid, the DNA fragment containing the genes encoding for the amylolytic enzymes together with the LEU2 selective marker region was amplified by PCR and cloned into the unique HpaI site within the rDNA region of the pJRD. In order to avoid integrating unnecessary antibiotic marker or vector sequences, the fusion gene and marker region flanked by the rDNA sequences was amplified by PCR from the pJBG plasmid and directly introduced into *S. cerevisiae* FY23Δpet191 cells by electroporation. Among

the integrants, the one with the highest amylolytic activity was selected and the advantages of using this strain for the single-step bioconversion of starch into ethanol were investigated under both shake flask and controlled bioreactor cultivation conditions by following the time dependent variations in starch utilization, glucose concentration, biomass formation and bioethanol production.

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10.
Saccharification of cellulosic biomass: Regulation of cellulase and xylanase activities under catabolic repression conditions

Oscar Ariel Rojas Rejón [1,*], Ma. Teresa Ponce Noyola [1], Hector M. Poggi Varaldo [1], Mayra de la Torre Martínez [2]

[1] *Cinvestav Ciudad de Mexico, Distrito Federal, Mexico*
[2] *Ciad Hermosillo, Sonora, Mexico*

Lignocellulose is the major structural component of cell wall in plants and represents the major source of renewable organic matter. Residual plant biomass can be converted into different products including microbial biomass, bio fuels, organic acids and carbon sources. Effective conversion of lignocellulose to fermentable sugars requires: size reduction, pre-treatment and enzymatic hydrolysis (Zhang et al., 2006). The cost of enzymes is one of the factors determining the economics of a biocatalytic process and it can be reduced finding optimum conditions for their production (Lynd et al., 2002). The mutant PN-120 of *Cellulomonas flavigena* after mutagenic treatment was isolated in continuous culture. This mutant exhibited 2.5 and 10 fold more xylanase and β-glucosidase activities respectively than the wild strain (Ponce-Noyola and de la Torre, 1995). However mutant PN-120 shows catabolic repression when glucose is added to culture media. A third mutagenesis step under repression conditions yields mutant PR-22 which increased 1.3 and 4 fold xylanase and CMCase activities, respectively. Mutant PR-22 was grown in saline medium, 1% sugar cane bagasse and 0.02% yeast extract in a batch reactor. Glucose and cellobiose were added at different concentrations to cultures growing at exponential growth phase in sugar cane bagasse, in order to analyze the behaviour of enzyme production and activities. Despite the concentration (20 mM) reached in the culture, xylanase activity did not show an important decrease as well CMCase. This result shows the nature of feedback resistance of mutant PR-22. When 10 mM and 15 mM of both sugars (glucose and cellobiose) were tested, the xylanase and CMCase activities did not show any change respect to the control (without repressor). In all cases the production of enzyme were diminished but not stopped. The complete saccharification of lignocellulosic residues requires the use of enzymes resistant to feed-back inhibition. Mutant PR-22 of *Cellulomonas flavigena* exhibited and important increase of enzyme activity even under catabolic repression conditions respect *C. flavigena wt*. The industrial use of PR-22 is possible since is a mutant resistant to high concentration of readily metabolized sugars.

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11.
Polyhydroxyalkanoate production in sugarcane—recognizing temporospatial complexity

Lars Nielsen

The University of Queensland, Brisbane, QLD Australia

Sugarcane has the potential to be a key crop in the biofactory revolution. It is the second fastest growing tropical grass, produces a large biomass, partitions carbon into sucrose at up to 42% of the dry weight of the stalk, has a mobile pool of hexose sugars through most of its life cycle, is difficult to get to produce viable seed and therefore is vegetatively propagated, and can be harvested multiple times before replanting. To test the ability of sugarcane to be a biofactory we chose to engineer into sugarcane plants the genetic pathway for poly-3-hydroxybutyrate (PHB).

The three gene pathway, phaA, phaB and phaC has been successfully engineered into a number of plant species. However, either levels of PHB accumulation were low or one or more of the products from the PHB biosynthetic pathway had adverse effects on the transgenic plants. We have targeted the products from the *Ralstonia eutropha* PHB biosynthetic pathway to several subcellular compartments of sugarcane.

In our initial trials, we found that the polymer accumulated in the leaves of chloroplast-targeted lines at levels up to 1.88% of dry weight and to 0.01% when the genes were targeted to the cytosol. No polymer was produced in lines harbouring the PHB biosynthetic genes targeted to mitochondria. We conducted a glasshouse trial with replicates of six independent lines of PHB-producing sugarcane. PHB production remained constant during