BIOREFINING MIXED SUGARS USING HIGH DENSITIES OF GROWTH-ARRESTED CORYNEBACTERIA

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Introduction

Research and development of renewable energies has recently regained prominence given anticipated shortages of fossil-fuel-based energies and parallel rising prices of fossil-derived fuels and chemicals. This changing economic landscape combines with environmental degradation to constitute powerful forces of change [36]. Particularly, the quantities of ethanol produced via biotechnological processes to displace petroleum as a transportation fuel are expected to dramatically increase in the coming years [8]. At present, the bulk of the industrial ethanol production is ensured via the fermentation of easily accessible sugars, for example derived from corn starch, sugar cane or sugar beet. The yeast *Saccharomyces cerevisiae* is the primary organism to ensure these fermentations [3]. However, the cost analysis of the existing production processes clearly demonstrates that their economic performances are tightly linked to the costs of fermentation raw materials [27], and thus indirectly to the amount –and limitation– of arable land that can be redirected for the production of these materials. Furthermore, the flattening experience-curve observed by industrial manufacturers suggests that radical innovation is needed to make bio-ethanol a competitive commodity compared to fossil fuels [35]. As a result, it becomes increasingly clear that the global scale implementation of ethanol as a primary transportation fuel will only become a reality once sugars can be extracted from abundant and easily accessible lignocellulosic biomass in a cost-effective manner [9, 26, 35]. However, current technological limitations of yeast-based processes include their inability to use the pentose xylose, a major component of typical lignocellulosic fractions, and their sensitivities to the presence of various growth inhibitors that are generated at the pre-treatment step of the saccharification process of lignocellulosic materials [16].

We have previously validated the concept of using a combination of very high cellular densities, oxygen deprivation, and decoupling of the cell generation phase and the product production phase of fermentation processes, as a means to induce conditions that are sub-optimal for cellular growth but optimal for producing an array of products [12-14, 32, 34, 35]. The process we developed is characterized by a minimal number of manufacturing steps and can be applied in continuous fermentations or batch fermentations with cell re-use. Moreover, it can be applied to anaerotolerant organisms, such as *Corynebacterium glutamicum*, or to quorum sensing sensitive organisms such as *Escherichia coli* [34]. However, *S. cerevisiae* is ill-suited for this process as, in this latter organism, growth and ethanol production are coupled phenomena [2]. What is more, yeast require traces of molecular oxygen for the synthesis of various vital compounds [21]. Nonetheless, this flexibility and simplicity in design represent important manufacturing advantages that combine with the proven robustness of numerous other industrial microorganisms, such as microorganisms used in the food industry, to enable bioconversions characterized by favorable economics [35]. It cannot be

overemphasized that the intrinsic robustness of the design we propose is a key feature for capturing synergies between various bioconversions. Particularly, this makes possible optimal manufacturing integration within a biorefinery framework. Furthermore, we have engineered the amino acid production workhorse *C. glutamicum* to produce ethanol and organic acids via continuous or semi-continuous processes that efficiently operate at very high cellular densities in the absence of concomitant cellular growth [12, 13]. Notably, the strain/bioprocess couples we developed using these technologies exhibit two critical attributes: on the one hand a reduced heat generation and a greatly reduced negative impact on yields of the various growth-inhibitors that are typically present in saccharified mixtures of lignocellulosic biomass materials, and on the other hand reduced by-products formation and reduced carbon source utilization for vegetative functions [14].

The next challenge to address in order to enable the cost-effective utilization of lignocellulosic biomass with this latter system is to confer to *C. glutamicum* the ability to catabolize in parallel glucose and xylose. *C. glutamicum* is equipped with a complete pentose phosphate pathway including glucose 6-phosphate dehydrogenase (a hetero-multimeric protein coded for by the genes *zwf* and *opcA*), 6-phosphogluconolactonase (*pgl*), 6-phosphogluconate dehydrogenase (*gnd*), transketolase (*tkt*), and transaldolase (*tal*) (Fig. 1) [39]. The genes *tkt*, *tal*, *zwf*, and *pgl* form a putative operon [39]. However, corynebacteria typically do not grow on xylose as a carbon source, as they lack the enzyme xylose isomerase, despite *C. glutamicum* harbors a *xylB* gene coding for xylulokinase [15].

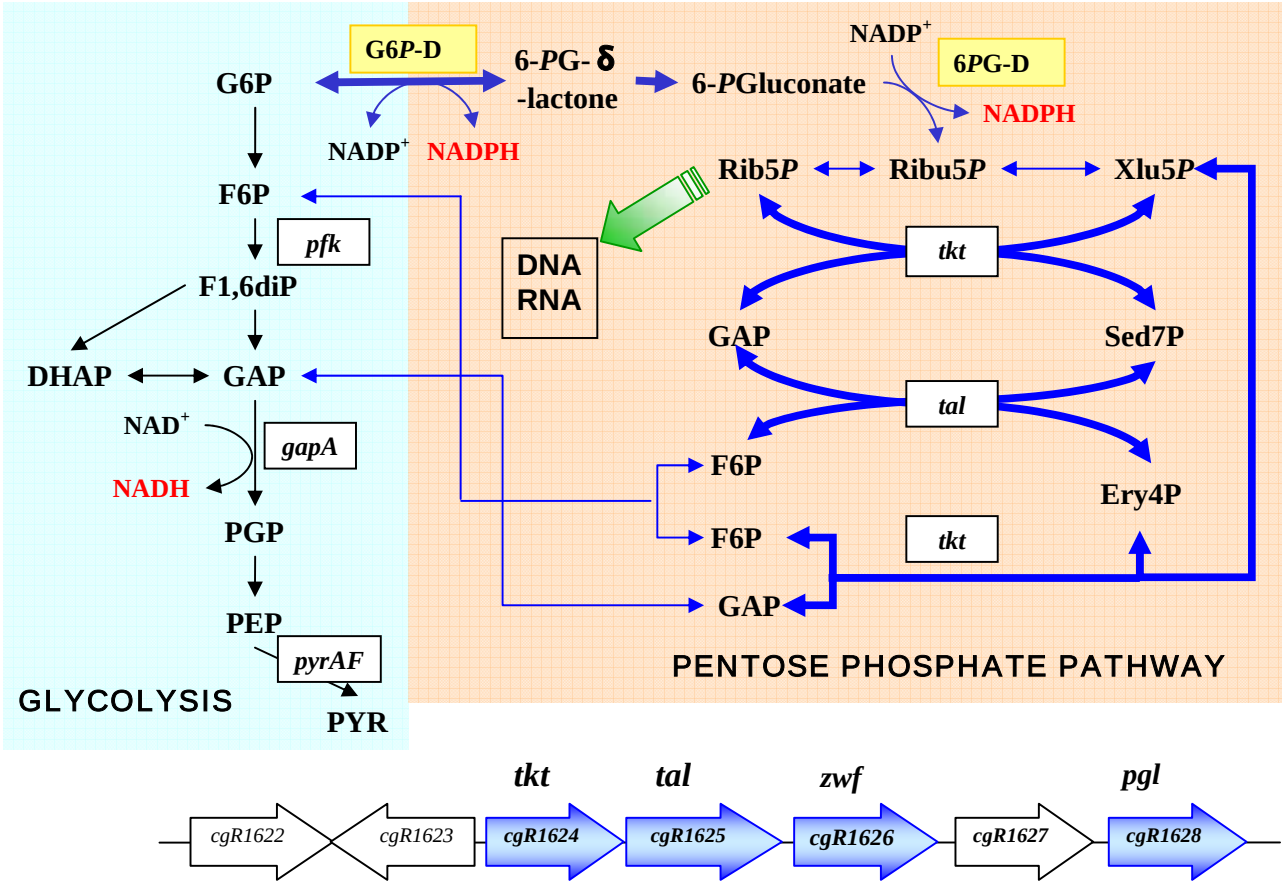

Fig. 1. The pentose phosphate pathway of *C. glutamicum* R. Like in all Corynebacterianeae sequenced to date, the pentose phosphate pathway genes (*tkt*, *tal*, *zwf*, *pgl*) are organized in a putative operon in *C. glutamicum* R [39]. In addition, a *xylB* gene coding for the enzyme xylulokinase is present in this strain as in several corynebacteria, including *C. glutamicum* ATCC 13032, *C. efficiens* YS-314, and *C. diphtheriae* NCTC 13129, but this gene is absent from the genome of *C. jeikeium* K411 [15]. The physiological role of the pentose phosphate pathway, which constitutes a glycolysis bypass, is to replenish pools of precursor metabolites (Rib5P, Ery4P) and reducing equivalents (NADPH). The high demand in NADPH for amino acid biosynthesis makes the pentose phosphate pathway central to numerous biotechnological applications of *C. glutamicum* [39]. G6P-DH shows decreased activity in cells grown on organic acids as opposed to cells grown on glucose [28]. This key enzyme that regulate the flux to the pentose phosphate pathway is chiefly controlled by the ratio [$NADP^+$]/[NADPH] [22, 39]. However, increased transketolase activity has been shown to be a successful strategy to increase the yield of amino acid production, such as tryptophan yields [10, 11]. DH: dehydrogenase, F6P: fructose-6-phosphate, GAP: glyceraldehyde-3-phosphate, Ery4P: erythrose-4-phosphate, Sed7P: sedoheptulose-7-phosphate, Rib5P: ribose-5-phosphate, Ribu5P: ribulose-5-phosphate, 6*P*Gδlactone: D-glucono-1,5-lactone 6-phosphate, 6*P*gluconate: 6-phospho-D-gluconate, G6P: glucose-6-phosphate, G6P: glucose-6-phosphate dehydrogenase, F1,6diP: fructose-1,6-bisphosphate, DHAP: dihydroxyacetonephosphate, PGP: glycerate-1,3-bisphosphate, PEP: phosphoenolpyruvate, PYR: pyruvate, *tkt*: transketolase, *tal*: transaldolase, *pfk*: phosphofructokinase, *gapA*: glyceraldehyde-3-phosphate dehydrogenase, *pyrAF*: pyruvate kinase. The gene nomenclature is conform to the annotation of the *C. glutamicum* R complete genome sequence (accession number AP009044) [40].

Materials and Methods

Bacterial strains, media, cultivation conditions and plasmids

C. glutamicum R is a wild type Gram-positive bacterium from the laboratory collection. It was isolated in Japan from a meadow soil sample. Strain R-CEL is an adaptive mutant of strain R that is able to grow on cellobiose as a sole source of carbon [18]. Strains ATCC 13032, ATCC 14297, ATCC 14751, ATCC 15136, ATCC 21065, and ATCC 21305 were obtained from the American Type Culture Collection. Strains CRX1, CRX2, and CRX3 are strain R derivatives harboring, respectively, plasmid pCRA810 (multicopy shuttle *E. coli-C. glutamicum* vector encoding *xylA* under control of the strong constitutive promoter P_{trc}) or pCRA11 (same as pCRA810 but encoding an additional fragment encoding *xylB* under P_{trc} control, see Fig. 1, CRX2: intact chromosomal *xylB*, CRX3: disrupted chromosomal *xylB*) [15]. The *dam*$^-$*dcm*$^-$ *E. coli* JM110 was used as a source of non-methylated plasmid DNA for the electrotransformation of *C. glutamicum* R [31]. For genetic manipulations, *E. coli* strains were grown at 37°C in Luria-Bertani medium [23]. For aerobic growth conditions, *C. glutamicum* R and the recombinant strains CRX1, CRX2 and CRX3 were pre-cultured at 33°C overnight in nutrient rich medium (A-medium) containing (per liter) 2 g urea, 2 g yeast extract, 7 g casamino acids, 7 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 6 mg $FeSO_4 \cdot 7H_2O$, 4.2 mg $MnSO_4 \cdot H_2O$, 0.2 mg biotin, 0.2 mg thiamine, and supplemented with 4%(w/v) glucose [13]. Where appropriate, media were supplemented with antibiotics. The final antibiotic concentrations were: for *E. coli* 50 μg ml^{-1} of ampicillin, 50 μg ml^{-1} of chloramphenicol; for *C. glutamicum* 5 μg ml^{-1} of chloramphenicol, and 50 μg ml^{-1} of kanamycin.

To investigate the growth performance of *C. glutamicum* under aerobic conditions, wild type and

recombinant strains CRX1, CRX2 and CRX3 were harvested by centrifugation (5,000 × *g*, 4°C; 10 min). Cell pellets were subsequently washed twice with mineral medium (BT-medium) containing (per liter) 7 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 6 mg $FeSO_4 \cdot 7H_2O$, 4.2 mg $MnSO_4 \cdot H_2O$, 0.2 mg biotin, 0.2 mg thiamine. After the second wash, cells were resuspended in 100 ml of BT-medium containing an appropriate concentration of sugars. The resulting mixture was incubated at 33°C with constant agitation (200 rpm) in a 500-ml flask.

Organic acid production under oxygen deprivation

For organic acid production, both wild type and recombinant CRX2 cells grown in standard aerobic-phase cultures were harvested by centrifugation (5,000 × *g*, 4°C; 10 min). Cell pellets were subsequently washed twice with mineral medium (BT-medium). After the second wash, cells were resuspended to give an appropriate cell concentration in 80 ml of BT-medium containing 100 mM sodium bicarbonate and were incubated at 33°C with constant agitation in a lidded 100-ml medium bottle. Organic acid production was started by adding variable amounts of sugar. The pH was monitored using a pH controller (DT-1023, Biott Co. Ltd., Japan) and maintained at 7.5 by supplementing the medium appropriately with 2.5 N ammonia.

DNA manipulations

Plasmid DNA was isolated either by the alkaline lysis procedure [23] or by using a HiSpeed Plasmid Midi kit (Qiagen Inc.) according to the manufacturer's instructions, modified, when extracting DNA from corynebacteria, by using 4 mg ml^{-1} lysozyme at 37°C for 30 min. Restriction endonucleases were purchased from Takara (Osaka, Japan) and used as per the manufacturer's instructions. PCR was performed using a GeneAmp PCR System (Applied Biosystems, Foster city, CA, USA) in a total volume of 100 µl with 50 ng of chromosomal DNA, 0.2 mM dNTPs, 2% dimethylsulfoxide in LA Taq polymerase buffer with $MgCl_2$ and 4 units of LA Taq polymerase (Takara) for 30 cycles at temperatures of 94°C for denaturation (1 min), 55°C for annealing (1 min), and 72°C for extension (2 min). Oligonucleotide primers used in this study are listed in Table 2. The resulting PCR fragments were purified with a QIAquick PCR purification kit (Qiagen, Inc.). Corynebacteria were transformed by electroporation as previously described [31]. Transformation of *E. coli* was performed by the $CaCl_2$ procedure [23]. All sequencing necessary to verify the constructs was performed by the dideoxy chain termination method as previously described [24] with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA) using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The nucleotide sequences of both strands were determined. DNA sequence data were analyzed with the Genetyx program (Software Development, Tokyo, Japan).

Construction of recombinant plasmids containing xylose metabolism genes

Construction of plasmids pCRA810 and pCRA811 was reported previously [15]. Briefly, the 1.4-kb *E. coli xylA* gene [25] was amplified using *E. coli* K-12 chromosomal DNA as a template and the oligonucleotide primers CTCTGAATTCACCTGATTATGGAGTTCAAT, including an *Eco*RI overhang, and CTCTCCCGGGCATATCGATCGTTCCTTAAA, including a *Sma*I overhang. The PCR amplicon was subsequently digested with the relevant enzymes and ligated to *Eco*RI and *Sma*I-digested pTrc99A plasmid DNA. The resulting construct contains the *xylA* gene under control of the strong constitutive *trc* promoter on a 1.6-kb *Bgl*II-*Bam*HI fragment. This fragment was subsequently amplified by PCR using the oligonucleotide primers CTCTAGATCTCCGACATCATAACGGTTCTG (*Bgl*II overhang) and CTCTGGATCCCTTCTCTCATCCGCCAAAAC (*Bam*HI overhang). After appropriate digestion, the resulting PCR product had *Bgl*II and *Bam*HI cohesive ends that were used for its cloning

into *Bgl*II and *Bam*HI digested pCRA1 plasmid DNA, yielding plasmid pCRA810. Similarly, the 1.6-kb *E. coli xylB* gene [19] was amplified by PCR using *E. coli* K12 chromosomal DNA as a template and the oligonucleotide primers CTCTGAATTCTTTAAGGAACGATCGATATG (*Eco*RI overhang) and CTCTCCCGGGTTCAGAATAAATTCATACTA (*Sma*I overhang). The resulting PCR product was subsequently digested with *Eco*RI and *Sma*I and ligated to *Eco*RI and *Sma*I-digested pTrc99A DNA. This new chimeric plasmid contains a 1.7-kb *Fba*I-*Bam*HI DNA fragment encoding the *xylB* gene under control of the *trc* promoter. This DNA segment was amplified by PCR using the oligonucleotide primers CTCTGGATCCCTTCTCTCATCCGCCAAAAC (*Bam*HI overhang) and CTCTTGATCAACCGACATCATAACGGTTCTG (*Fba*I). The resulting PCR product was used to ligate the amplicon to *Bam*HI circularized pCRA810 plasmid DNA, yielding plasmid pCRA811. The restriction map of plasmid pCRA811 is given in Fig. 2. *C. glutamicum* R was transformed by electroporation with either pCRA810 or pCRA811 plasmid DNA. Transformants were selected on the basis of chloramphenicol resistance and subsequently screened for growth on xylose as the sole carbon source. For each plasmid, one of these clones able to metabolize xylose was isolated to purity to yield strains CRX1 and CRX2. Strain CRX3 was constructed by electrotransformation using plasmid pCRA810 DNA where the native *xylB* gene had been inactivated by Tn*5* transposon mutagenesis [15].

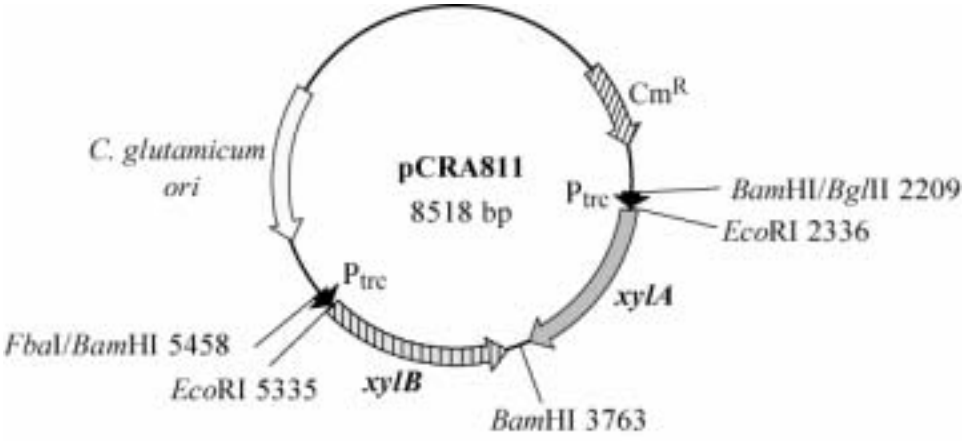

Fig. 2. Map of Plasmid pCRA811. The *E. coli – C. glutamicum* shuttle plasmid pCRA811 contains in opposite orientation the *xylA* and *xylB* genes under control of the strong and constitutive promoter P_{trc}. The corynebacterial origin of replication is from the rolling circle plasmid pBL1 [29], the *E. coli* expression vector pTrc99A (Pharmacia) was used as the main backbone of the plasmid. Digestion by *Eco*RI conveniently cleaves *xylA* and *xylB* on a 3.0-kb promoterless cassette.

Enzymatic assays

Cell-free extracts obtained from batch experiments samples were used for assaying enzyme activities that were measured at 340 nm and 30°C in a final volume of 1.0 ml using a Beckman DU800 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). Cultures were harvested by centrifugation at 5,000 × *g*, 4°C; 10 min. Cell pellets were washed once with extraction buffer (100 mM Tris-HCl (pH7.5), 20 mM KCl, 20 mM $MgCl_2$, 5 mM $MnSO_4$, 0.1 mM EDTA, and 2 mM DTT). The resulting cell suspensions were sonicated using an ultrasonic homogenizer (Astrason model XL2020, Misonix, USA) in an ice water bath for three 2-min periods, interrupted by 2-min cooling intervals. Cell debris was removed by centrifugation (20,000 × *g*, 4°C; 30 min). The cell lysates thus produced were

subsequently used as crude extracts for enzyme assays. 1 U of enzyme activity was defined as the amount of activity necessary to convert 1 μmol of NADH to NAD^+ per min. Protein concentrations were determined using a Bio-Rad protein assay kit. Lactate dehydrogenase (LDH) assays were performed as previously described [4]. Xylose isomerase activity was determined based on NADH oxidation by sorbitol dehydrogenase as previously described [7]. Xylulokinase assays were performed as reported elsewhere [6].

Analytical procedures

Samples were centrifuged (10,000 × *g*, 4°C; 10 min) and the resulting supernatants were analyzed for the presence of sugars and organic acids. Organic acids concentrations were determined by high-performance liquid chromatography using an apparatus (8020, Tosoh Corporation, Tokyo, Japan) equipped with an electric conductivity detector and a TSKgel OApak-A column (Tosoh Corporation, Tokyo, Japan) operating at 40°C with a 0.75 mM H_2SO_4 mobile phase at a flow rate of 1.0 ml min^{-1}. Sugar concentrations were determined by high-performance liquid chromatography using an apparatus (8020, Tosoh Corporation, Tokyo, Japan) equipped with a refractive index detector and a TSKgel Amide-80 column (Tosoh Corporation, Tokyo, Japan) operating at 85°C with an 80% acetonitrile mobile phase at a flow rate of 1.0 ml min^{-1}. Cell mass was determined by measuring the absorbance at 610 nm (A_{610}) using a spectrophotometer (DU800, Beckman Coulter Inc. CA, USA). An A_{610} of 1 corresponded to 0.39 mg dry weight cell ml^{-1}.

Results and Discussion

Sugar utilization spectrum of various C. glutamicum strains

While corynebacteria are saprophytic organisms commonly recovered from soil, dairy products, and plant materials [20], they exhibit a relatively narrow spectrum of sugar utilization (Table 1). This limitation is particularly exemplified by the lower number of secondary carriers for sugar transport exhibited by these bacteria, as compared to *E. coli* or to bacteria of the *Bacillus-Clostridium* branch of the eubacteria [40]. As a result, in order to enable the cost-effective utilization of lignocellulosic materials for the production of a variety of commodity compounds, it is critical to engineer novel catabolic pathways in this organism. Particularly, xylose and arabinose constitute the main pentoses of typical lignocellulosic hydrolysates [38], with xylose representing 5 to 20% and arabinose 1 to 5%. Notably, corynebacteria, though they are unable to grow on xylose as a sole carbon source, encode a functional *xylB* gene but lack *xylA* to permit xylose catabolism [15].

Strain	Glucose	Mannose	Galactose	GlcUA	Cellobiose	Xylose	Arabinose
R	+	+	-	+	-	-	-
R-CEL	+	+	-	+	+	-	-
ATCC 13032	+	+	-	+	-	-	-
ATCC 14297	+	+	-	+	-	-	-
ATCC 14751	+	+	-	+	-	-	-
ATCC 15136	+	+	-	+	-	-	-
ATCC 21065	+	+	-	-	-	-	-
ATCC 21305	+	+	-	-	-	-	-

Table 1. Sugar utilization spectrum of various *C. glutamicum* strains. The sugar utilization spectrum of various *C. glutamicum* strains was verified by pre-culturing all strains aerobically until late log-phase in nutrient rich medium (A-medium) supplemented with 40 g/l glucose. The resulting cell cultures were used to inoculate mineral medium containing 40 g/l of the indicated sugars. +: growth; -: no growth. GlcUA: glucuronic acid.

Engineering of a xylose catabolism pathway in C. glutamicum R

C. glutamicum R was transformed by electroporation with either plasmid pCRA810 or pCRA811 to generate derivatives that are able to grow on xylose as the sole carbon source (Fig. 3), as follows: CRX1 (harbors pCRA810 containing a P_{trc}-*xylA* construct and a wild type *xylB* gene on the chromosome); CRX2 (harbors pCRA811 containing a P_{trc}-*xylA* and P_{trc}-*xylB* construct and a wild type *xylB* gene on the chromosome); CRX3 (harbors pCRA810 containing a P_{trc}-*xylA* construct, and a disrupted wild type *xylB* gene on the chromosome). It is noteworthy that strain CRX2, which encodes a recombinant *xylB* gene in addition to *xylA*, grew faster on xylose than strain CRX3 that relies on the native *xylB* gene for xylulokinase. However, the specific consumption rates were not significantly different (Fig. 3). All strains grew faster on glucose, and at the same specific growth rate of $\mu = 0.28\ h^{-1}$, than on xylose (e.g., CRX2 grew on xylose at a 40% lower specific growth rate). Interestingly, the corynebacterial *xylB* gene or its gene product appear to be subject to induction in the presence of xylose, as demonstrated by the observation that in CRX3 xylulokinase specific activity is 9-fold higher when cells are grown on xylose (0.44 U/mg of protein) than on glucose (0.05 U/mg of protein). This phenomenon is masked in strain CRX2, suggesting that such regulation acts at the transcriptional level, rather than at the translational or protein modification level.

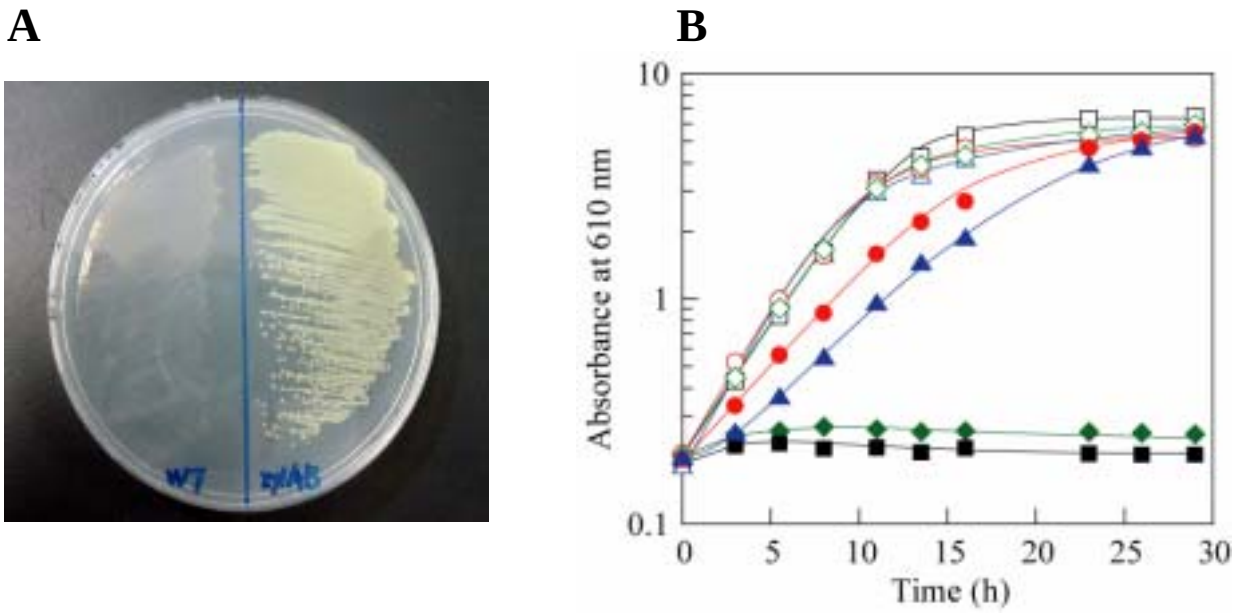

Fig. 3. Mutant *C. glutamicum* able to grow on xylose as a sole carbon source. Panel A: *C. glutamicum* R transformed with plasmid pCRA811 encoding *xylA* and *xylB* under control of the strong constitutive promoter P_{trc} is able to grow on agar mineral medium supplemented with 40 g/l xylose as a sole carbon source, while the wild type only shows residual growth if any. WT: wild type; XYLAB: CRX2. Panel B: Comparative aerobic growth of *C. glutamicum* strains in liquid mineral medium containing either glucose (open symbols) or xylose (closed symbols). Wild type *C. glutamicum* R (squares) and CRXI (triangles), CRX2 (circles), and CRX3 (diamonds) were first grown aerobically to late log phase in A medium containing 40 g/l glucose. These pre-cultures were subsequently used to inoculate mineral medium, containing either 20 g/l glucose or 20 g/l xylose, to an initial absorbance at 610 nm of 0.2. The graph was plotted using data collected from three independent experiments where each point had been measured in triplicates.

To investigate whether glucose and xylose are utilized in parallel by strain CRX2, this recombinant organism was incubated in mineral medium containing an equimolar mixture of the two sugars (Fig. 4). Wild type *C. glutamicum* R was used as a control. As expected, *C. glutamicum* R ceased growth upon depletion of the xylose pool, whereas the recombinant CRX2 consumed both glucose and xylose completely. Nevertheless, and as observed in the previous experiment, the maximum specific glucose consumption rate of CRX2 cells was higher (2.8 mM $h^{-1}g^{-1}$) than that of xylose (1.5 mM $h^{-1}g^{-1}$). It is worth noting that despite xylose consumption was apparently facilitated once the glucose pool had been depleted, thereby suggesting the presence of phenomena limiting xylose uptake or utilization in the presence of glucose, CRX2 cells grew to high cell densities without undergoing any diauxic shift. This observation suggests that in addition to the constitutive expression of the *xylA* and *xylB* genes, all the other necessary components for xylose utilization are present even in the presence of glucose, from proteins involved in xylose transport and metabolism to enzymes of the pentose phosphate pathway.

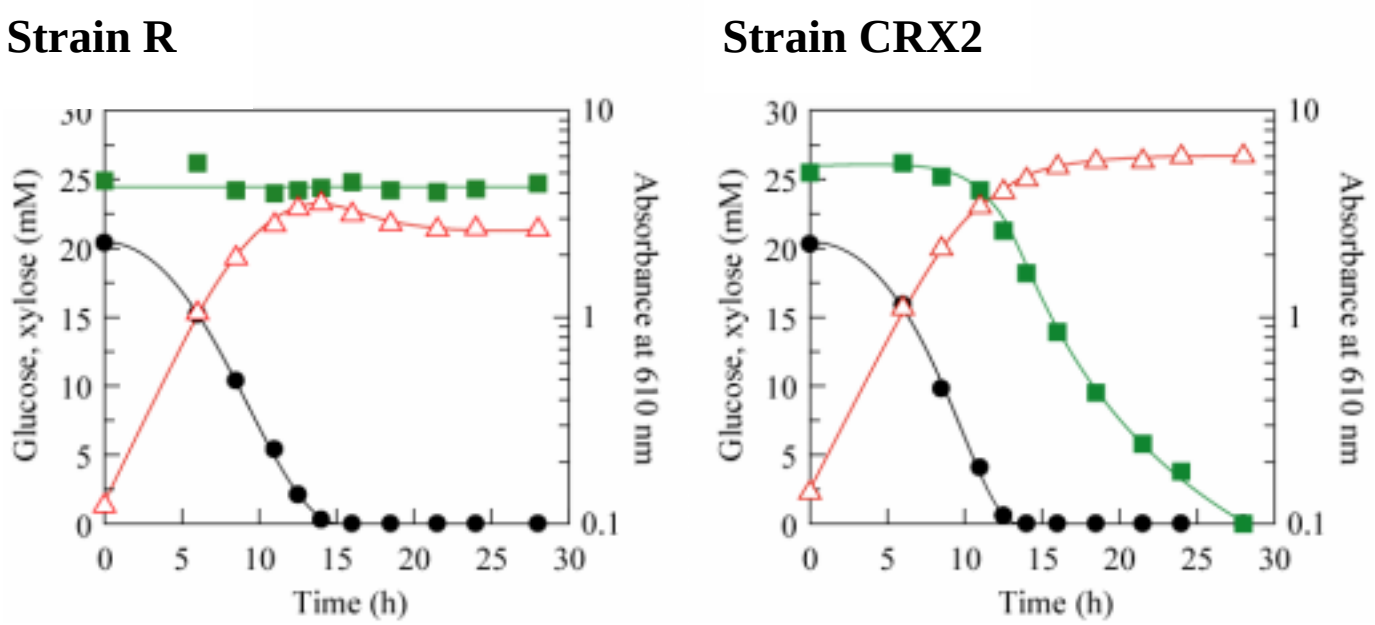

Fig. 4. Aerobic growth of wild type *C. glutamicum* R and of its xylose-catabolizing recombinant CRX2. Left panel: wild type *C. glutamicum* R. Right panel: strain CRX2 harbors the *xylA*- and *xylB*-expressing pCRA811 plasmid, in addition to the native corynebacterial *xylB* gene. Both strains were pre-cultured to late log phase in A medium containing 40 g/l glucose and subsequently used to inoculate mineral medium containing 3.6 g/l glucose (20 mM) and 3.6 g/l xylose (20 mM), to an absorbance at 610 nm of 0.2. Closed circles: glucose concentration, closed squares: xylose concentration, open triangles: cell density as measured by the absorbance at 610 nm. The graphs were plotted using data collected from three independent experiments where each point had been measured in triplicates.

Organic acid production by growth-arrested cells

We have developed a core bioconversion process which is based on the control of vegetative growth. Control of cell division can be achieved by different means, including key nutrient starvation [30] or genetic control of fundamental growth-related genes such as *ftsZ* [1, 5, 17]. We have recently revisited the effect of oxygen deprivation on the growth of *C. glutamicum* and observed that *C. glutamicum* ceases to grow (but does not undergo autolysis) when it is incubated in media lacking a suitable terminal electron acceptor, while it maintains active many of its metabolic pathways. Combined with very high cellular densities, such conditions enable the control of the growth of anaerotolerant organisms, such as *C. glutamicum*, and of quorum sensing sensitive organisms such as *E. coli* (Fig. 5). Importantly, both of these conditions are straightforward and can be inexpensively implemented at the industrial scale, where cells can be concentrated via centrifugation, and oxygen deprivation conditions can be quickly reached by the simple absence of aeration.

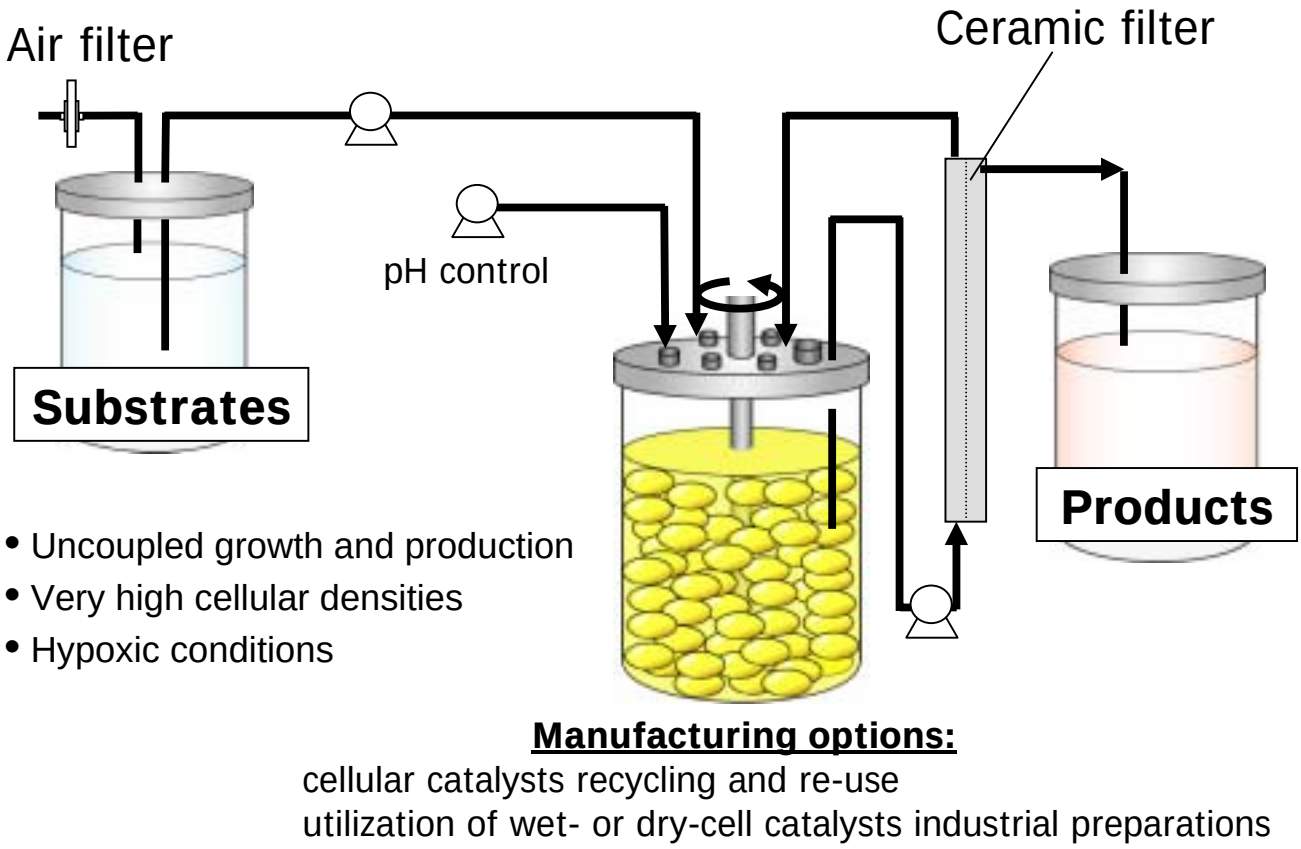

Fig. 5. Growth-independent bioconversion process using *C. glutamicum*. In this core process, cell catalysts production phase and product production phases are uncoupled. Growth cessation is achieved by a combination of two factors: very high cellular density and oxygen-deprivation. These conditions address additional objectives that are important manufacturing issues: very high cell catalyst densities lead to higher yields, while operating under oxygen-deprivation conditions alleviates the need to ensure high oxygenation throughout the fermenter and triggers typical anaerobic metabolism that is suitable for the production of useful compounds such as organic acids or ethanol. As a result, this process design is applicable in principle to any anaerotolerant or quorum sensing sensitive microorganism. The key observation is that under growth cessation conditions, the main metabolic machineries of numerous microorganisms remain active. This design confers several important industrial attributes: 1) substrates consumption by cells for vegetative function is greatly reduced leading to higher conversion rates; 2) there is a linear relationship between cell density and yield; 3) the process is simple, easy to implement, applicable to numerous microbial genera, and it comprises only a limited number of manufacturing steps; 4) conditions suboptimal for microbial growth but optimal for product production can be used thus reducing the impact of the presence of growth-inhibitors; 5) fast on/off responses are observed upon substrate addition or depletion; 6) the fermentation heat generation rate is constant thus simplifying the implementation of continuous operations and leading to reduced utility usage [14]. Moreover, the uncoupling of the cell catalyst growth phase and of the product production phase enables to re-use cells

several times or to prepare inocula off-site in order to benefit from out-sourcing the catalyst production phase.

We have previously successfully applied this industrial process design to produce both organic acids and ethanol using various recombinant strains of *C. glutamicum* (Fig. 6) [12-14]. We verified the ability of the novel recombinant strain CRX2 to produce organic acids from xylose using high cellular density and oxygen-deprivation process conditions. Strain CRX2 completely utilized both sugars and produced higher levels of organic acids than the wild type (Fig. 7).

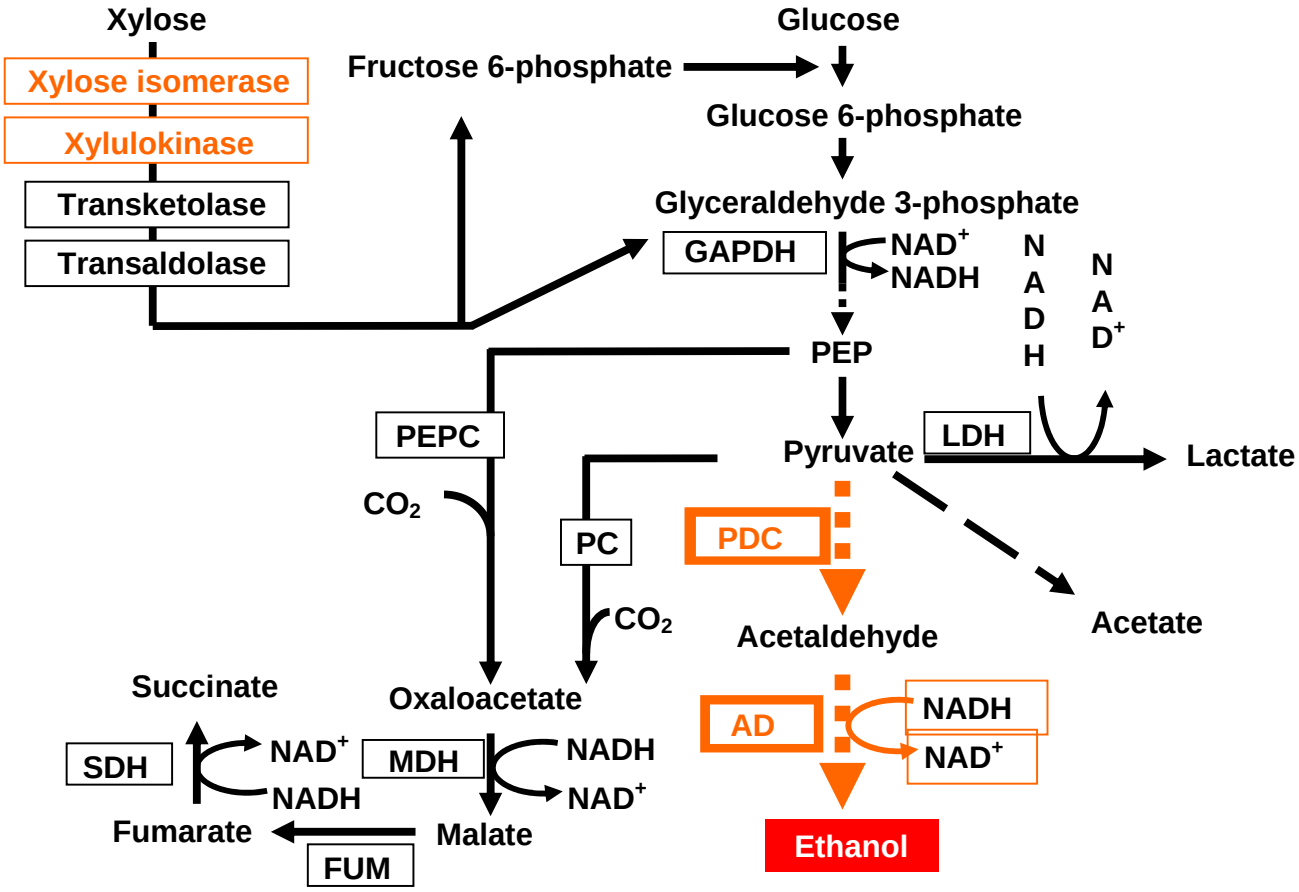

Fig. 6. Recombinant metabolism of *C. glutamicum* for ethanol and organic acid production. The cloning of xylose isomerase and xylulokinase in the genome of *C. glutamicum* R enables the creation of novel commodity chemical producers, for example by cloning the *pdc* and *adh* genes from *Zymomonas mobilis* [12]. Furthermore, metabolic engineering techniques can be applied to increase the versatility of these converters to include for example both ethanol and organic acids production capabilities. For example, the *ldh* gene could be disrupted to limit by-product formation during ethanol manufacturing operations from a *pdc-adh* expressing *C. glutamicum* strain [12] or during succinate production from a wild type *C. glutamicum* strain [13]. Likewise, *pepc* could be disrupted to direct most of the carbon flow

towards lactate production [13]. Notably, the ability to tightly control the genetic expression of key metabolic genes such as *ldh* or *pepc* would provide very flexible manufacturing options from the same industrial strain. Building such optionality upstream in development of novel microbial fermentors is important for enabling maximum flexibility during industrial operations, since the enhanced flexibility thus attained would facilitate management of production campaign schedules and logistics issues.

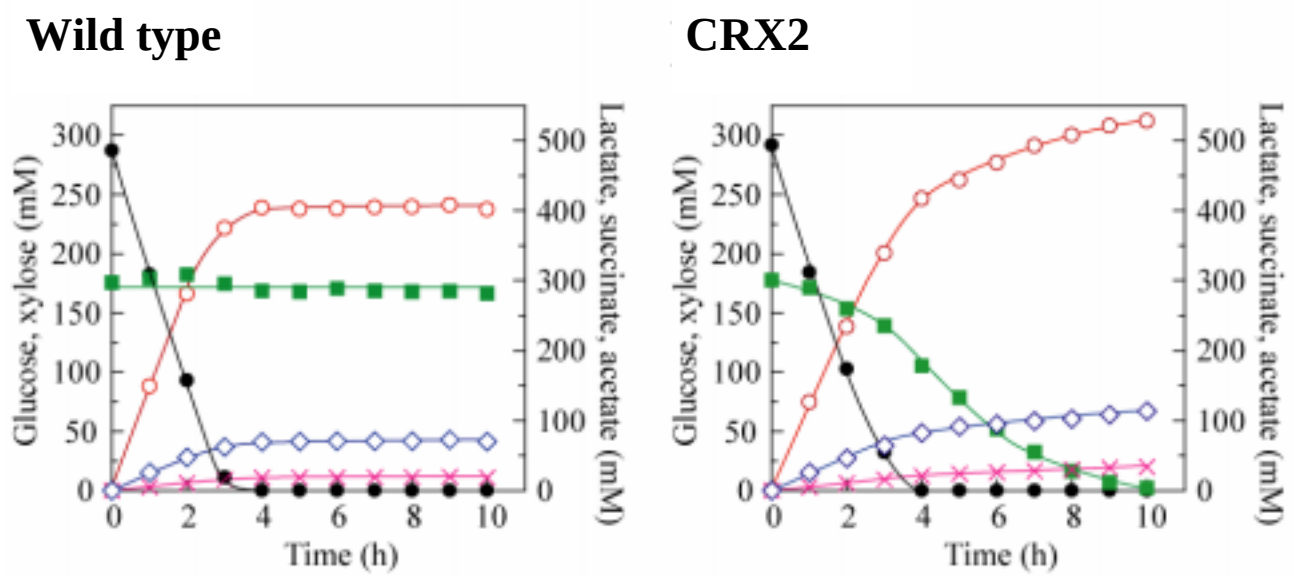

Fig. 7. Organic acid production from glucose-xylose mixtures by *C. glutamicum* CRX2. Production of organic acids by CRX2 cells was verified from glucose-xylose mixtures. Wild type *C. glutamicum* R was used as a control. Both strains were first aerobically grown to late log phase in A medium containing 40 g/l glucose and the appropriate antibiotic for plasmid segregational stability. Cells were harvested and subsequently used to inoculate mineral medium containing both 50 g/l glucose and 25 g/l xylose in the presence of 200 mM bicarbonate to a final cell concentration of 20 g dry weight cells per liter. The 2/1 glucose/xylose ratio mimics that observed in typical lignocellulosic biomass hydrolysates. Sodium bicarbonate was added to ensure a pump-priming effect to increase the NAD^+/NADH ratio [13]. Closed squares: xylose; closed circles: glucose; open circles: lactate; open diamonds: succinate; crosses: acetate. The data represent the averages calculated from data collected from three independent experiments where each point was measured in triplicates.

It is important to emphasize that no diauxic phenomenon was observed during the course of the numerous experiments performed and thus that all the enzymes necessary for xylose metabolism are present throughout the fermentation, but that the xylose specific consumption rate of strain CRX2 is 1.4 fold higher when cells are pre-cultured on xylose as compared to glucose. However, and as expected considering the cloning strategy employed, activities of xylose isomerase and xylulokinase are similar in both cases (respectively, 0.27 to 0.31 U/mg of protein and 10 to 11 U/mg of protein). Furthermore, addition of glucose to xylose-metabolizing CRX2 cells under hypoxic conditions leads to an immediate decrease in the specific xylose consumption rate (Fig. 8). Together, these observations reinforce the view that the various factors that impact the metabolism of xylose in CRX2 cells mainly act either at the

sugar transport level or downstream of the xylose catabolism mediated by the *xylA* and *xylB* gene products. These phenomena would thus need to be studied in light of both advances in corynebacterial pentose transport systems and global metabolic regulation. It is worth noting that these mechanisms might include novel regulatory mechanisms that do not have any precedent in the classical model bacteria *E. coli* and *B. subtilis*. Detailed understanding of these mechanisms is a pre-requisite to the further improvement of corynebacteria-mediated bioconversions [33, 37].

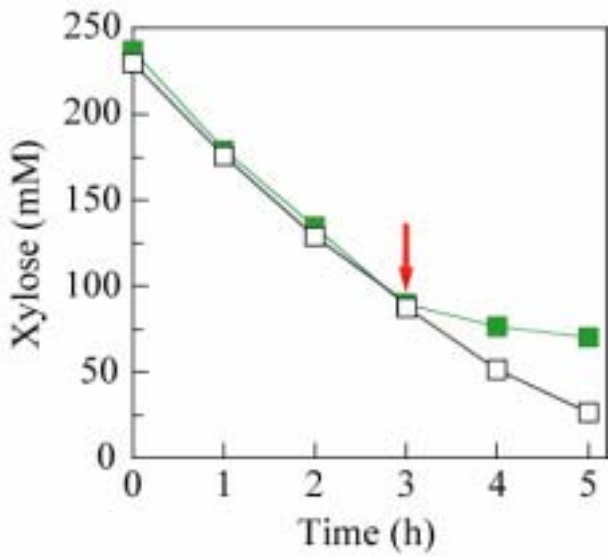

Fig. 8. Effect of glucose addition on the xylose metabolism of *C. glutamicum* CRX2 incubated under oxygen-deprivation conditions. CRX2 cells were pre-cultured aerobically to late log phase in A medium supplemented with 40 g/l xylose and the relevant antibiotic to ensure plasmid segregational stability. Cells were subsequently harvested by centrifugation and used to inoculate mineral media containing 36 g/l xylose at a final cell concentration of 10 g dry weight cells per liter. Xylose concentrations were measured with (closed symbols) or without (open symbols) glucose addition. As indicated by the arrow, after 3 h of reaction, glucose was spiked to 15 g/l in one of the cultures. The data represent the averages calculated from data collected from three independent experiments where each point was measured in triplicates.

A look forward

The results presented here demonstrate the feasibility of engineering *C. glutamicum* by recombinant DNA technology to utilize a larger spectrum of sugars, and to use fermentation technology to produce via this organism a variety of products typically derived from fermentative metabolism. The ability to produce organic acids without growth and without diauxic effect, even when using a mixture of sugars, is an important attribute for the production from lignocellulosic biomass of ethanol and other commodity chemicals. Continued engineering of this organism to use numerous carbon sources, such as starch, cellobiose, xylose, or the other sugars typically contained in lignocellulosic biomass will enable to generate highly cost-effective bioconverters. Combined with the fermentation technology we describe,

these research efforts pave the way to the development of novel bioconversions that constitute, given their intrinsic flexibility, radical innovation options for the realization of the integrated biorefinery vision. While the industrial objective is to produce a large array of commodity products and fine chemicals, the ultimate economic objective is to address the needs of multiple markets and thus drive the development of these market by initiating a new wave in the technology cycle of applied biology by validating not only new process concepts, but also new products and applications.

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