THERMOPHILIC GRAM-POSITIVE BIOCATALYSTS FOR BIOMASS CONVERSION TO ETHANOL

DE-FC36-01GO11073, A000

FINAL TECHNICAL PROGRESS REPORT
December 2003

Recipient Organization: **University of Florida**
Department of Microbiology and Cell Science
Box 110700
Gainesville, FL 32611

Contact Person: **K. T. Shanmugam, Professor**
Phone: **352-392-2490**
Fax: **352-392-5922**
e-mail: **shan@ufl.edu**

FINAL TECHNICAL PROGRESS REPORT

DOE Grant No.: DE-FC36-01GO11073, A000
Title: Thermophilic Gram-positive Biocatalysts for Biomass Conversion to Ethanol

Co-PIs: K. T. Shanmugam, L. O Ingram, Julie A. Maupin-Furlow, J. F. Preston and H. C. Aldrich

Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611

Task 1. Develop a functional PET operon for model Gram-positive bacteria
Task 2. Physiological and genetic characterization of selected strains from the enrichment
Task 3. Metabolic engineering of selected strains for ethanol production

PHYSIOLOGICAL AND GENETIC CHARACTERIZATION OF SELECTED STRAINS FROM THE ENRICHMENT

During this grant period, we have completed the analysis of over 400 bacterial strains isolated from 77 environmental samples as potential second generation biocatalysts which can grow and ferment under conditions that are also optimal for the activity of commercial fungal cellulases (pH 5.0 and 50°C). Selected isolates are expected to carry out simultaneous saccharification and fermentation (SSF) of cellulose in a cost-effective manner. All of these isolates were evaluated for the following physiological properties to identify the best candidates for detailed analysis.

1. Ability to grow under anaerobic conditions
2. Ability to grow and ferment a variety of sugars; glucose, xylose, arabinose, galactose, mannose, sucrose, cellobiose, etc.
3. Ability to grow in minimal-salts medium with and without corn steep liquor
4. Ability grow and ferment sugar cane bagasse hemicellulose hydrolysate

Based on the results of these experiments, four isolates were selected for detailed study. Isolates 17C5 and 36D1 grew and fermented the sugars in sugar cane bagasse hemicellulose hydrolysate as well as SSF of crystalline cellulose, Solka Floc, in minimal-salts medium with 1% corn steep liquor. Isolate P4-102B was easily transformable by plasmid DNA as a prelude to metabolic and genetic engineering. Isolate P4-74B was included because of its growth and fermentation characteristics.

Taxonomy of the new isolates

Based on the sequence of first 500 bp of the 16S rRNA sequence, 37 of the 39 tested isolates, including the four selected strains, were found to form a unique phylogenetic group with the nearest neighbor being *Bacillus coagulans*. One isolate, strain Y58 was found to be *B. smithii* while the last tested isolate, 57H2, was closely related to *B. smithii*. To confirm these identities, the DNA encoding the entire 16S rRNA from three of the isolates, 17C5, 36D1 and P4-102B, was sequenced and these

sequences were compared to other sequences in the rRNA sequence database. Based on full length sequence also, the isolates 17C5, 36D1 and P4-102B formed a unique phylogenetic group with the nearest neighbor being *Bacillus coagulans* although the three strains were initially isolated from different geographical locations within the country (Fig. 1). As presented in this Figure, bacteria identified as *B. coagulans* form a very diverse phylogenetic group and except for one strain of *B. coagulans*, strain IDSp, other bacteria identified as *B. coagulans* in the database are phylogenetically distinct from these new isolates. The *B. coagulans* ATCC type strain, ATCC 7050, also differed from the new isolates by its inability to utilize xylose. The second generation bacterial isolates may constitute a new species of *Bacillus*; in this study, they are referred as *Bacillus* spp. in accordance with their unique phylogeny. The ability to produce lactic acid as the major fermentation product and the phylogenetic grouping with *B. coagulans* indicates that the second generation biocatalysts are part of a larger sporogenic lactic acid bacteria group.

Fermentation of Glucose

Detailed growth and fermentation profiles of four of the selected second generation isolates on glucose are presented in Table 1 and Fig. 2 and Fig. 3. Since ethanologenic second generation biocatalysts are yet to be constructed, lactic acid production was used to establish the general physiological and fermentation characteristics of these biocatalysts. Once the appropriate ethanologenic constructs are made, the basic lactate fermentation properties are expected to be readily transferrable to ethanol production. In these experiments, the cultures were grown in home-made fermentation units at 50°C and a constant pH of 5.0, maintained by addition of 2N KOH. Cultures were mixed by a magnetic stirrer bar at 200 RPM. The gas phase above the 250 ml of liquid in a 500 ml vessel was air. Inoculum for these experiments was grown under aerobic conditions at 50°C in LB+glucose (1%) (LB; tryptone, 1%; yeast extract, 0.5%; NaCl, 0.5%) to mid-exponential phase of growth.

After a very short lag (less than 2 hours), LB+glucose cultures grew in a linear manner until the maximal cell density was reached in about 12 hours (Fig. 2). Glucose utilization and lactate production followed the growth of the biocatalyst and continued until all the sugar (30 g/L) was exhausted from the medium. A culture maintained under strict anaerobic conditions with an argon gas phase grew very slowly suggesting that the initial build-up of cell mass required micro-aerobic conditions. Continued fermentation of glucose by the biocatalyst was independent of the gas phase.

Cell yield of strains 17C5 and P4-74B were significantly higher in rich medium with glucose than the other two strains although the cell density of strains 17C5 and P4-74B decreased significantly when they reached stationary phase (Fig. 2). All 4 cultures reached the same final yield of lactic acid and the time for complete fermentation of the added glucose was directly proportional to the highest observed cell density of the cultures with strain 17C5 fermenting 30 g/L glucose in about 16 hours with the highest volumetric productivity of lactate (2.5 g L^{-1} h^{-1}).

All four strains grew in glucose-minimal medium supplemented with 1% corn steep liquor with strain P4-74B growing at the highest growth rate (Fig. 3). Strain 36D1 had the second highest growth rate and reached the stationary phase by about 24 hours. Strain 17C5 had the lowest growth rate for reasons unknown. However, the final cell yield in glucose-minimal medium was about the same for

all four strains. The amount of time required to completely ferment the 30 g/L glucose depended on the growth rate and cell yield and varied between 48 and 96 hours. Corn steep liquor was essential for optimum growth of the organism in minimal medium.

The main fermentation product of all four strains from glucose was lactate (Table 1). Acetate and ethanol accounted for about 5% of the total products produced irrespective of the medium composition. In rich medium, strains 36D1 and P4-74B had the highest specific glucose consumption rate and corresponding lactate production rate. In minimal medium, the specific rate of glucose consumption and lactate production were about the same for strains 36D1, P4-74B and P4-102B. Total product yield from glucose was about 85%. Based on glucose fermentation in both rich and minimal media, strains 36D1 and P4-74B appear to be the most efficient.

Fermentation of Xylose.

Xylose is the primary sugar in the hemicellulose fraction of hardwood and agricultural residues such as sugar cane bagasse, corn fiber, corn stover, straw, etc. All four isolates used in this detailed study, strains 17C5, 36D1, P4-74B and P4-102B, grew and fermented xylose in both rich medium and minimal-salts medium supplemented with corn steep liquor (Table 2; Fig. 4 and Fig. 5). Following various lengths of lag period, all four strains grew in LB+xylose linearly. Specific xylose consumption rate and lactate production rate were highest with strain 36D1 cultured in rich medium with strain P4-102B as the second highest (Table 2). In xylose-minimal-medium, the specific xylose consumption rate and lactate production rate of strains 17C5 and 36D1 were comparable and were the highest of the four select isolates. The level of acetate and ethanol among fermentation products varied between 9 and 18 % depending on the strain and medium. Strain 17C5 had the lowest level of these co-products (about 10%) while strain 36D1 had the highest level (about 18%). This reduced the lactate yield of strain 36D1 to 68% of the expected value in xylose-minimal medium although the total product yield of the two strains were comparable at 85%.

In minimal medium strain 36D1 was most effective in fermenting xylose converting 30 g/L xylose in less than 48 hours (Fig. 5). It is interesting to note that this strain required an additional 24 hours to ferment the same 30 g/L xylose in rich medium due to lower growth rate (Fig. 4). Based on these fermentation profiles, strain 36D1 emerged as the most effective glucose and xylose fermenting biocatalyst, especially in minimal salts medium supplemented with only corn steep liquor (volumetric productivity of 0.6 g L^{-1} h^{-1}). Increasing the sugar concentration to 50 g/L marginally increased the lactic acid yield beyond that with 30 g/L sugar, probably due to inhibition of fermentation by lactic acid. Production of lactic acid at a concentration higher than 0.4 M (about 35 g/L) was found to progressively inhibit fermentation (see the section on fermentation of hemicellulose hydrolysate).

The lactic acid produced by the four isolates was found to be L(+)-isomer with D(-)-isomer contributing to less than 4% of the total (Table 3).

Xylose utilization pathway

Many of the lactic acid bacteria used at the industrial level do not ferment pentoses such as xylose.

The few lactic acid bacteria capable of fermenting pentoses, such as *Lactobacillus pentosus, Lb. arabinosus*, etc. utilize phosphoketolase pathway for pentose utilization. The key enzyme of this pathway, phosphoketolase, cleaves xylulose-5-phosphate in the presence of inorganic phosphate to one molecule each of glyceraldehyde-3-phosphate and acetyl phosphate. The products of pentose fermentation by these bacteria are an equimolar amount of lactic acid and acetic acid plus ethanol. The loss of 2/5 of xylose carbon to acetyl phosphate will reduce the amount of xylose carbon that can be channeled to ethanol in the future ethanologenic constructs by about 40%.

The main product produced by the isolated second generation biocatalysts is lactic acid (about 80 to 90 % of fermentation products). Acetic acid and ethanol represented only 10-20% of the products produced from the pentose xylose suggesting that these biocatalysts utilize an alternate pathway, the pentose phosphate pathway, for xylose fermentation. In order to confirm that the pentose-phosphate pathway is used by the second generation biocatalysts for xylose metabolism, we determined the distribution pattern of C1-carbon of xylose into fermentation products since glyceraldehyde-3-phosphate directly yields pyruvate and products derived from pyruvate, lactate, acetate and ethanol. During the cleavage of xylulose-5-phosphate by phosphoketolase, carbon at 1-position of xylose is the C-2 carbon of acetate and ethanol. The lactic acid carbon skeleton is derived from the carbons 3-5 of xylose and in an organism with phosphoketolase pathway $^{13}C_1$-label in xylose will not be found in lactate. If the pentose-phosphate pathway is the main pathway by which the pentose is metabolized, 2/5 of the C3-carbon of glyceraldehyde-3-phosphate will be derived from C1-carbon of xylose while 1/5 of the C1-carbon of glyceraldehyde-3-phosphate will originate from C1-carbon of xylose. The presence of ^{13}C-label in lactate will confirm the metabolism of xylose through the pentose-phosphate pathway.

For these experiments, we used $^{13}C_1$-xylose and followed the products produced by strains 36D1 and P4-102B by ^{13}C-NMR. A typical ^{13}C-NMR spectrum obtained with the $^{13}C_1$-xylose fermentation products of strain 36D1 is presented in Fig. 6. Carbon 3 of lactate had the highest amount of ^{13}C originating from C1-xylose. Carbon 1 of lactate also carried significant amount of ^{13}C. The C2 position of lactate is not expected to originate from C1-position of xylose except for a small amount derived by randomization of carbon by the pentose-phosphate pathway. With C2 of lactate as a reference, C1 of lactate was enriched by about 5-fold and the C3 of lactate was enriched by about 17-fold by ^{13}C (Table 4). These results clearly show that the pentose phosphate pathway is the main pathway of xylose utilization in these second generation biocatalysts. Since pyruvate carbon is enriched with ^{13}C, the acetate and ethanol are also expected to carry ^{13}C-label. The ^{13}C-label was found only at the C2 position of acetate and ethanol. The inability to detect ^{13}C in C1 of acetate and ethanol is due to the small amount of ^{13}C-label entering the C2-position of pyruvate (contributing to C1 of acetate and ethanol) combined with the low concentration of these two compounds in the fermentation broth. Small amount of ^{13}C-label was detected in formate with non-growing cells indicating that pyruvate formatelyase is responsible for the acetate and ethanol produced by strain 36D1.

The presence and operation of pentose phosphate pathway in these biocatalysts is significant since all the xylose carbon will be routed through pyruvate. This supports complete recovery of xylose carbon as ethanol by decarboxylation of pyruvate to acetaldehyde and further reduction to ethanol in engineered second generation biocatalysts. Xylose metabolism through phosphoketolase is expected to yield one glyceraldehyde-3-phosphate and one acetyl phosphate leading to production

of one ethanol from pyruvate with at least 40% of xylose carbon lost as acetate.

Simultaneous Saccharification and Fermentation of Crystalline Cellulose

The main objective of this study is to isolate biocatalysts that can optimally grow and ferment under conditions that are also optimal for commercial fungal cellulases. The optimal conditions reported for fungal cellulases are pH 5.0 and 50°C. The biocatalysts we have isolated and characterized grew and fermented both hexoses and pentoses at 50°C and pH 5.0. All four selected biocatalysts were found to be competent in SSF of crystalline cellulose, Solka Floc. Since strain 36D1 fermented both glucose and xylose effectively in minimal salts medium with corn steep liquor, this strain was used to evaluate the SSF characteristics of these second generation biocatalysts.

In the first set of experiments, SSF of Solka Floc (2%; 117 mM glucose equivalent with a 5% moisture content) was carried out in minimal salts medium with 1% corn steep liquor with 15 FPU/g glucan of fungal cellulases (Spezyme CE; generously provided by Genencor) at 50°C and pH 5.0 (Fig. 7). The amount of lactate and other products produced from 2% Solka Floc over a 96 hours period was determined. From these values, volumetric productivity of the major product, lactic acid, was calculated. Volumetric productivity represents indirectly the rate at which cellulases are releasing glucose for growth and fermentation. In all of these SSF experiments, the inoculum size was 5% to minimize free glucose accumulation in the medium. The amount of free glucose in the medium was less than 3 mM during the first 24 hours of SSF indicating that the sugar released by the cellulases was consumed rapidly by the biocatalyst. Under these conditions, product inhibition of cellulase activity is expected to be minimal.

At 15 FPU/g glucan cellulase level, lactate production started after a lag of about an hour and was linear for about 18 hours. Small amount of acetate and ethanol were also produced between 6 and 12 hours of fermentation. After about 36 hours, lactate production reached a slow phase and continued at this low rate past 96 hours. Volumetric productivity of lactate was 6.2 mmol L^{-1} h^{-1}, the same as that of free glucose fermentation in minimal salts medium (Table 1). The product yield from cellulose at 96 hours of 180 mM is 77% of the expected maximum. Lactic acid accounted for about 78% of the products. The amount of free glucose in the medium during the first 24 hours was about 2.5 mM. However, glucose was not detected after 24 hours of fermentation indicating that the remaining cellulose was not effectively hydrolyzed. When fresh Solka Floc was added to the fermentation at 72 hours, lactic acid production resumed indicating that the enzymes were still active. These results suggest that only about 80 % of the cellulose fibers in autoclaved Solka Floc is accessible to cellulases under the conditions of the experiment.

In order to determine the minimum amount of cellulase required for optimal SSF, fermentations were carried out at different cellulase concentrations (Fig. 8). Volumetric productivity of lactate as well as the total product yield increased linearly with cellulase concentration from 0 to about 5 FPU/g glucan (Fig. 8). Increasing the Spezyme concentration beyond this level led to a smaller rate of increase in volumetric productivity until about 30 FPU/g glucan was reached. Under these SSF conditions, the highest volumetric productivity of 7.1 mmol L^{-1} h^{-1} was reached at about 30 FPU/g glucan and this value is less than 2-fold higher for a 6-fold increase in enzyme concentration from 5 to 30 FPU/g glucan. At 15 FPU/g glucan, the volumetric productivity was 84% of the value with 30

FPU/g glucan. Total product yield at 96 hours was about 199.5 ± 5 mM between the cellulase levels of 7.5 and 30 FPU/g glucan. These results show that an increase in cellulase level from 15 to 30 FPU/g glucan only increased the volumetric productivity by 1.12-fold without any change in either lactic acid or total product yield. In subsequent SSF, a spezyme CE concentration of 15 FPU/g glucan was used.

The optimal pH for the SSF of Solka Floc using strain 36D1 was between 5.0 and 5.5 (Fig. 9). Although the volumetric productivity of lactate was highest at pH 5.0, product yield was maximum at pH 4.5 reaching 85% of the theoretical yield after 96 hours of SSF (Table 5). Apparently, the optimum pH for SSF of cellulose by strain 36D1 is between 4.5 and 5.0. The amount of acetate and ethanol produced by the culture also increased with increasing culture pH.

At a cellulase concentration of 15 FPU/g glucan and at pH 5.0, the rate of SSF of cellulose by strain 36D1 was highest at 55°C. Although the product yield did not significantly change between 43°C and 55°C, the volumetric productivity of lactate was about 2-times higher at 55°C than the 4.3 mmol L^{-1} h^{-1} at 43°C (Fig. 10).

Results of these SSF clearly show that the second generation biocatalyst, strain 36D1, fermented the sugars released by Spezyme CE under the conditions that appear to be optimal for the enzyme activity.

Fermentation of Sugar Cane Bagasse Hemicellulose Hydrolysate

The sugar cane bagasse hemicellulose acid hydrolysate was generously provided by BC International. This hydrolysate had a total sugar concentration of 81.6 g/L with xylose accounting for 86.5% of the total. Small amount of glucose (11.5 g/L) and arabinose (1.2 g/L) were also present in the hydrolysate. The hydrolysate was adjusted to pH 5.0 with calcium hydroxide. The resulting calcium sulfate was removed by centrifugation and the supernatant was used in fermentations. When all the four isolates were tested for their ability to ferment sugar cane bagasse hemicellulose hydrolysate, only strains 17C5 and 36D1 fermented hemicellulose hydrolysate at a concentration of 25% in mineral salts medium with 0.5% corn steep liquor. Increasing the hydrolysate concentration to 50% led to inhibition of fermentation.

To minimize inhibition, the hemicellulose hydrolysate was over-limed with calcium hydroxide and the final pH was adjusted to 5.0. In preliminary experiments with over-limed hemicellulose hydrolysate, strain 17C5 was found to be an effective biocatalyst and was used in further experiments involving over-limed hydrolysate.

Fermentations were conducted using three levels of total sugar: 256 mM (Fig. 11A), 412 mM (Fig. 11B), and 483 mM (Fig. 11C). In all fermentations, glucose and arabinose were metabolized first followed by xylose. Fermentation profiles were generally similar for all three levels of sugar although fermentation times increased with substrate. With 256 mM sugar (40 g L^{-1}), lactate production was measurable after 8 hours and fermentation was completed within 120 h. With 412 mM sugar (60 g L^{-1}), fermentation proceeded at a constant rate until the lactate concentration reached about 0.4 M (36 g L^{-1} lactic acid). Complete fermentation of all sugars in this fermentation to 617 mM lactate

(55.5 g L^{-1}) required an additional 144 hours due to a progressively declining fermentation rate. With the highest level of sugar tested (483 mM; 72 g L^{-1}), 78 mM xylose remained after 192 h of incubation. These results suggest that fermentation is inhibited by lactate concentrations above 0.4 M. Even at the highest sugar concentration of 483 mM (about 72 g L^{-1}) lactate titer did not increase beyond 0.6 M (54 g L^{-1}), consistent with 617 mM (55.8 g L^{-1}) lactate (Table 6) representing a near upper limit for strain 17C5 at pH 5.0 (50°C) in this medium. Irrespective of the initial sugar concentration, the lactic acid produced by strain 17C5 was L(+)-lactic acid at an optical purity higher than 99%.

Lactate yields were calculated based on sugar utilized and ranged from 0.9 g lactate per g sugar for the lower two sugar concentrations to 0.86 g lactate per g sugar for the highest sugar concentration (Table 6). Maximal volumetric rate of sugar metabolism was determined to be 5.5 mmol xylose L^{-1} h^{-1} (approximately 0.8 g sugar L^{-1} h^{-1}).

Simultaneous Saccharification and Co-fermentation

In the next set of experiments strains 17C5 and 36D1 were evaluated for their ability to ferment sugar cane bagasse hemicellulose hydrolysate (over-limed) and Solka Floc (cellulose) simultaneously. Results of these experiments are presented in Fig. 12. Strain 17C5 fermented all the sugars in the hydrolysate and most of the sugars released from cellulose by about 96 hours yielding about 400 mM lactate (36 g/L). SSCF continued at a lower rate past 192 hours when the experiment was terminated. The rate of SSCF by strain 36D1 was slightly lower than strain 17C5.

These results show that these new biocatalysts are capable of fermenting both the xylose-rich hemicellulose hydrolysate and cellulose simultaneously and with minimum amount of commercial cellulase.

DEVELOP A FUNCTIONAL PET OPERON FOR MODEL GRAM-POSITIVE BACTERIA

Cloning *pdc* from *Sarcina ventriculi*

As a first step towards isolating a pyruvate decarboxylase gene that can be expressed in a Gram-positive organism such as the second generation biocatalyst, a degenerate oligonucleotide was synthesized based on the N-terminal amino acid sequence of the *S. ventriculi* PDC protein for use as a probe to identify the *pdc* gene. This approach facilitated the isolation of a 7.0-kb *Bcl*I genomic DNA fragment from *S. ventriculi*. The fragment was further subcloned and the sequence of a 3,886 bp *Hinc*II DNA fragment revealed an open reading frame (ORF) of 1,656 bp encoding a protein with an N-terminus identical to that of the *S. ventriculi* PDC protein. This ORF was designated *pdc*. A canonical Shine-Dalgarno sequence is present 7 bp upstream of the *pdc* translation start codon. In addition, a region 82 to 110 bp upstream of *pdc* has limited identity to the eubacterial –35 and –10 promoter consensus sequence. Downstream (43 bp) of the *pdc* translation stop codon is a region predicted to form a stem-loop structure followed by an AT-rich region, consistent with a rho-independent transcription terminator. Thus, the *S. ventriculi pdc* appears to be transcribed as a monocistronic operon like the *Z. mobilis pdc* gene.

Production of *S. ventriculi* PDC Protein

Unlike *Zymomonas mobilis pdc*, the codon usage of the *pdc* of *S. ventriculi* was different from that of *E. coli*. In particular, the *pdc* gene of *S. ventriculi* required elevated use of $tRNA_{AUA}$ and $tRNA_{AGA}$ both of which are relatively rare in *E. coli*. This is in contrast to the *Z. mobilis pdc* gene, which does not use the AUA codon and has only minimal use of the AGA codon. This suggests that the production of *S. ventriculi* PDC protein in recombinant *E. coli* may be limited by mRNA translation. Even in an *E. coli* strain that is expressing the rare tRNAs required by the *Sarcina pdc*, the level of PDC activity produced by the recombinant *E. coli* was only about 0.16 U per mg protein at 5 mM pyruvate in the cell extracts.

Because of the low level of expression of the *Sarcina pdc* in *E. coli*, we utilized a Gram-positive bacterial host *Bacillus megaterium* WH320 for construction of the PET operon containing appropriate *pdc* and *adh* genes. A *B. megaterium/ E. coli* shuttle plasmid (pWH1520) with xylose inducible *xylA* promoter was used as a plasmid vector in these experiments.

A *Bsp*EI-*Xba*I fragment containing *Sarcina pdc* gene was cloned into appropriately digested plasmid vector pWH1520. The resultant *B. megaterium* expression plasmid pJAM420 carried the *S. ventriculi pdc* gene, along with the Shine-Dalgarno sequence and T7 transcriptional terminator of the original pET21d vector. The *pdc* gene was positioned to interrupt the *B. megaterium xylA* gene *(xylA')* of plasmid pWH1520 and to generate a stop codon within *xylA'*. The Shine-Dalgarno sequence of the inserted *pdc* gene was positioned directly downstream of the *xylA'* stop codon to allow for translational coupling in which the ribosomes would terminate at the stop codon for *xylA'* and then reinitiate at the *pdc* start codon. Plasmid, pJAM420, was used for expression of *S*vPDC in *B. megaterium*.

Expression of the *Sarcina pdc* was induced in *B. megaterium* with xylose, and the *S*vPDC protein was purified from this host. The high level expression of the *pdc* gene in this Gram-positive host yielded about 8.35 mg protein from 15 g of cells (wet wt.). This is in contrast to purification of *S*vPDC from *E. coli*, which only yielded 0.2 mg purified protein from 14.8 g of cells. The purified *S*vPDC from *B. megaterium* was a 235 kDa homotetramer of 58 kDa subunits as determined by Superdex 200 gel filtration chromatography and SDS-PAGE.

The recombinant *S*vPDC from *B. megaterium* displayed sigmoidal kinetics. The recombinant *S*vPDC from *B. megaterium* had a *K*m of 3.9 mM for pyruvate and a Vmax of 98 U per mg of protein when assayed at pH 6.5 and room temperature. When assayed at the optimal conditions, 32°C and pH 6.72, an increase in both *K*m (6.3 mM for pyruvate) and Vmax (172 U per mg protein) was observed. The PDC was also heat stable retaining about 95% of the activity after 90 min incubation at 60°C at pH 5.0 to pH 5.5. These results show that the *Sarcina ventriculi* PDC can be produced at high level in a Gram-positive host in an active form and can function at 50°C.

Gram-positive Portable Ethanol Operon (PET).

For successful production of ethanol from pyruvate, both PDC and alcohol dehydrogenase (ADH) need to be produced at optimal levels. A plasmid containing the *S. ventriculi pdc* gene and the *adh*

gene from *Geobacillus stearothermophilus* was constructed using plasmid pWH1520 that was successfully used for expression of *pdc* in *B. megaterium*. The resulting portable ethanol (PET) plasmid, pJAM423 (Fig. 13), was transformed into *B. megaterium*. After xylose induction, a significant fraction of cell cytoplasm was composed of the *S. ventriculi* PDC and *G. stearothermophilus* ADH proteins (Fig. 14). In preliminary experiments, the amount of ethanol produced by the *B. megaterium* with plasmid pJAM423 was about twice (20 mM) of the bacterium without the plasmid. These results show that the PET operon is functional in *B. megaterium* but high level ethanol production needs further genetic and metabolic engineering.

Gene transfer in second generation biocatalyst

Among the four isolates tested, strain P4-102B was readily transformable by an *E. coli*/Gram-positive bacteria shuttle vector, pNW33N, plasmid DNA isolated from a restriction/modification minus *E. coli*. Chemical transformation of protoplasts was not as effective as electroporation.

Towards metabolic engineering of these biocatalysts, we also isolated the genes coding for D-lactate dehydrogenase (d-*ldh*) and pyruvate formate lyase (*pfl*). Experiments are in progress to isolate the l-*ldh* gene. These DNA, after appropriate deletion, will be introduced into the host to remove the native lactate production system and the acetate production pathway to achieve homoethanol production in the presence of *S. ventriculi pdc* gene.

These results show that appropriate DNA as well as methods for introduction of PET operon into the second generation biocatalysts have been developed. Due to limitations in funding, construction of ethanologenic second generation biocatalysts is for the future.

Table 1. Fermentation profile of 3% glucose in LB medium and in minimal medium with 1% corn steep liquor at pH 5.0 and 50°C

Isolates	Medium	Cell mass g/L	Glucose utilized mM	Lactate mM	Acetate mM	Ethanol mM	Glucose consumption mmol L^{-1} h^{-1}	Volumetric productivity mmol L^{-1} h^{-1} (Lactate)	Specific productivity mmol g^{-1} h^{-1} (Lactate)	Specific glucose consumption rate mmol g^{-1} h^{-1}	Yield (%) Lactate	Yield (%) Total
17C5	LB	2.14	173.23	278.25	9.36	6.92	17.46	28.29	30.54	18.85	80.31	85.10
	MM	1.18	144.19	209.80	1.85	8.14	2.96	4.87	22.88	13.90	72.75	76.47
36D1	LB	1.42	169.30	278.58	4.86	5.13	5.86	9.63	44.50	27.08	82.27	85.37
	MM	1.34	162.50	271.05	5.88	6.35	3.76	6.23	29.79	17.99	83.40	87.27
P4-74B	LB	2.17	166.49	274.42	12.35	6.04	11.72	19.16	41.48	25.37	82.41	87.99
	MM	1.28	161.13	261.94	5.05	4.68	5.15	8.91	31.81	18.39	81.28	84.35
P4-102B	LB	1.18	171.15	280.43	5.05	5.58	3.72	7.87	33.22	15.69	81.93	85.61
	MM	1.17	166.61	268.70	3.35	4.87	2.80	4.60	28.00	17.03	80.64	83.57

LB- Rich medium
MM- Minimal medium

Table 2. Fermentation profile of 3% xylose in LB medium and in minimal medium with 1% corn steep liquor at pH 5.0 and 50°C

Isolates	Medium	Dry Cell mass g/L	Xylose utilized mM	Lactate mM	Acetate mM	Ethanol mM	Xylose consumption mmol L^{-1} h^{-1}	Volumetric productivity mmol L^{-1} h^{-1} (Lactate)	Specific productivity mmol g^{-1} h^{-1} (Lactate)	Specific xylose consumption rate mmol g^{-1} h^{-1}	Yield (%) Lactate	Yield (%) Total
17C5	LB	2.25	199.78	253.84	9.20	20.61	7.54	9.16	11.48	9.45	76.24	85.65
	MM	1.65	200.82	256.07	11.60	14.60	4.79	5.10	11.67	10.96	76.51	84.83
36D1	LB	1.48	203.10	240.82	11.21	24.23	4.90	5.63	16.61	14.47	71.14	81.84
	MM	1.81	201.81	228.67	16.60	34.91	6.21	6.79	13.02	11.91	67.98	84.57
P4-74B	LB	2.49	190.89	229.97	28.41	22.96	7.51	9.03	7.06	5.87	72.28	88.92
	MM	1.83	201.79	245.87	22.38	16.88	4.97	5.91	7.45	6.26	73.11	84.97
P4-102B	LB	2.11	190.36	241.15	8.20	40.49	7.75	9.58	15.04	12.17	76.01	92.20
	MM	0.99	181.27	225.15	8.26	6.15	2.19	2.62	9.57	7.99	74.53	80.21

LB- Rich medium
MM- Minimal medium

Table 3. Isomeric form of lactic acid produced by second generation biocatalysts

Strain	concentration (mM)		Ratio	
	L (+)	D (-)	L/D	D/L
Glucose - minimal medium				
17C5	13.40	0.00	1.00	0.00
36D1	7.80	0.35	0.96	0.04
P4-74B	11.60	0.40	0.97	0.03
P4-102B	13.90	0.00	1.00	0.00
LB + glucose medium				
17C5	13.00	0.35	0.97	0.03
36D1	10.60	0.00	1.00	0.00
P4-74B	13.30	0.35	0.97	0.03
P4-102B	14.30	0.40	0.97	0.03

Glucose concentration was 3% in both media. Lactic acid isomer was determined by HPLC using a chiral column.

Table 4. ^{13}C-enrichment ratios for fermentation products produced from $^{13}C_1$-xylose.

Product	Carbon Position	Isotope Enrichment Ratio				
		Strain 36D1		Strain P4-102B		*E. coli* W3110
		Non-growing	Growing	Non-growing	Growing	
Lactate	C-1	4.5	4.9	5.6	5.1	10.8
	C-2	1.0	1.0	1.0	1.0	1.0
	C-3	15.9	17.3	12.6	13.2	11.3
Acetate	C-1	1.0	1.0	1.0	1.0	1.0
	C-2	10.2*	14.7*	4.8*	9.1	4.6
Ethanol	C-1	1.0	1.0	1.0	1.0	1.0
	C-2	39.0*	10.0	15.2	11.1	5.8

Second generation biocatalyst, strain 36D1 or P4-102B, was grown in LB+Xylose to mid-exponential phase in a pH-stat at pH 5.0 at 50°C. For the experiment with non-growing cells, 40 ml of culture was centrifuged and the cells were washed with 5.0 ml of LB. The cells were resuspended in 4.75 ml of LB-xylose (1%). Enough $^{13}C_1$-xylose was added to the culture to bring the xylose concentration to 1.2% and the ^{13}C-enrichment to 20.8%. For the experiment with growing cells, cells from 0.5 ml of the mid-exponential phase culture were removed from the pH-stat, washed with equal volume of LB, and resuspended in 4.75 ml of LB-Xylose medium. Both fermentations were carried out at 50°C with manual addition of 1.0 N KOH to maintain the pH between 6.0 and 7.0. When acid production stopped, cells were removed by centrifugation and the supernatant was subjected to HPLC for product analysis and also to ^{13}C-NMR for identification of the ^{13}C-enrichment. $^{13}C_1$ propionate (50 mM) served as a reference.

For *E. coli* W3110, 20 ml of cells were grown under anaerobic conditions in LB+Xylose until late-exponential phase at 37°C. Cells were collected by centrifugation, washed once with LB and resuspended in 5.0 ml of LB-Xylose with ^{13}C-enrichment. Fermentation was carried out at 37°C with manual pH control between 6.0 and 7.0.

All enrichment ratios were based on the natural abundance of ^{13}C at the indicated positions with C-2 of lactate and C-1 of acetate and ethanol as reference. *represents that the C_1-carbon of acetic acid and ethanol was not labeled or the amount of label at the C1- position was below the detection limit. The presented value was computed based on the sensitivity of the instrument for ^{13}C.

Table 5. SSF profile of strain 36D1 in mineral salts medium at different pH and temperature

pH	Temp. (°C)	Fermentation products (mM)					Volumetric productivity of Lactate (mmol $L^{-1}h^{-1}$)	% Yield	
		Lactate	Acetate	Succinate	Formate	Ethanol		Lactate	Total
4.5	50	182.76	7.55	1.45	0.00	8.95	5.54 ± 0.16	82.24	90.32
5.0	50	141.25	14.59	1.80	15.32	22.04	6.17 ± 0.09	63.56	80.86
5.5	50	113.92	27.61	4.22	58.15	29.53	5.96 ± 0.45	51.26	78.88
6.0	50	66.66	36.55	3.54	90.66	34.83	4.05 ± 0.24	30.00	63.71
7.0	50	4.89	19.57	0.37	17.42	7.11	0.87 ± 0.14	2.20	14.37
Temp. (°C)	pH								
30	5.0	122.24	12.20	1.62	0.00	7.70	1.50 ± 0.04	55.00	64.70
37	5.0	137.98	9.05	1.73	0.00	19.68	2.50 ± 0.14	62.09	75.80
43	5.0	146.03	11.53	1.53	11.96	24.78	4.32 ± 0.08	65.71	82.74
50	5.0	141.25	14.59	1.80	15.32	22.04	6.17 ± 0.09	63.56	80.86
55	5.0	152.94	11.97	1.31	0.00	12.23	8.66 ± 0.08	69.82	80.30
60	5.0	105.95	12.39	0.60	0.00	2.35	7.22 ± 0.16	47.68	54.58

* Batch fermentation was carried out for 96hrs.

Table 6. Sugar cane bagasse hemicellulose hydrolysate fermentation by *Bacillus* sp. strain 17C5[a]

Sugar[b] (mM)	Sugar consumed (mM)			Net Production (mM)[c]					Lactate Yield (%)[d]
	Glucose	Xylose	Arabinose	Lactate	Acetate	Ethanol	Formate	Succinate	
256	32.5 ± 1.6	224.5 ± 9.8	4.5 ± 0.4	403.7 ± 5.6	7.0 ± 1.2	2.5 ± 0.5	7.8 ± 2.6	4.8 ± 0.8	90
412	50.8 ± 1.2	349.1 ± 9.9	5.5 ± 0.3	617.4 ± 18.4	0.6 ± 0.6	5.2 ± 1.0	9.5 ± 4.5	7.7 ± 0.4	89
483	60.3	340.4	4.7	600.2	1.0	3.9	11.1	9.1	86

[a] Fermentations at three concentrations of total sugar (50°C and pH 5.0). Averages with standard deviations are based on three independent fermentations. A single fermentation was conducted with the highest sugar concentration, 483 mM.
[b] Sugar concentration at the beginning of fermentation.
[c] Lime-treated sugar cane bagasse hemicellulose hydrolysate contained 66 mM acetate. Corn steep liquor at 0.5% final concentration in the fermentations contained 5.5 mM lactate, 0.2 mM acetate and 0.025 mM succinate. Appropriate amounts of these compounds were subtracted to obtain the net production by the biocatalyst. Carbon recovery as products (excluding cells) averaged 90%.
[d] Product yield was calculated as a percentage of the maximum theoretical yield assuming 2 lactates per glucose and 1.67 lactates per pentose.

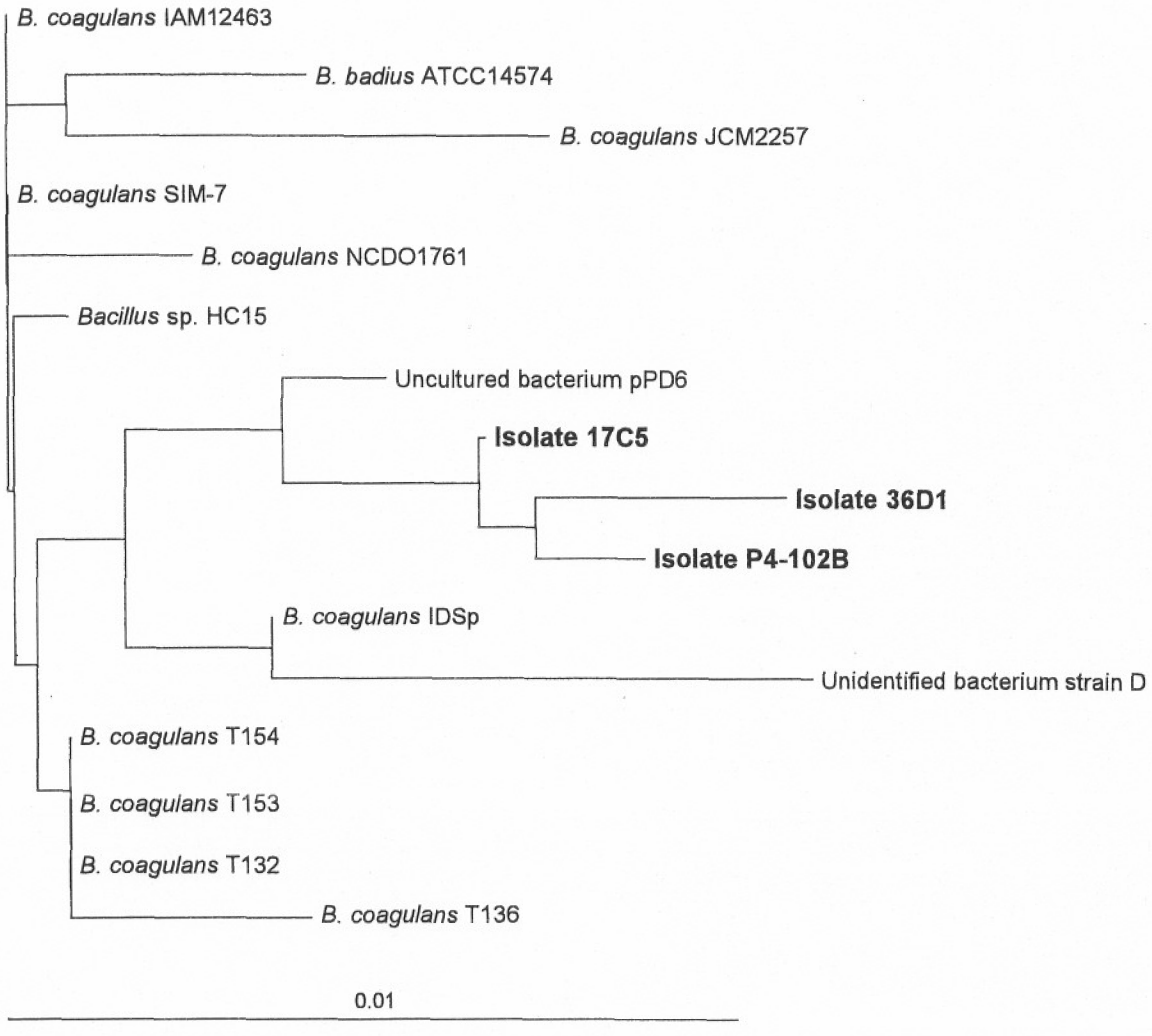

Fig. 1. Phylogenetic relationship of selected second generation biocatalysts to *Bacillus coagulans* and other closely related bacteria based on sequence of DNA coding for 16S rRNA.
The bar represents 1% divergence between sequences.

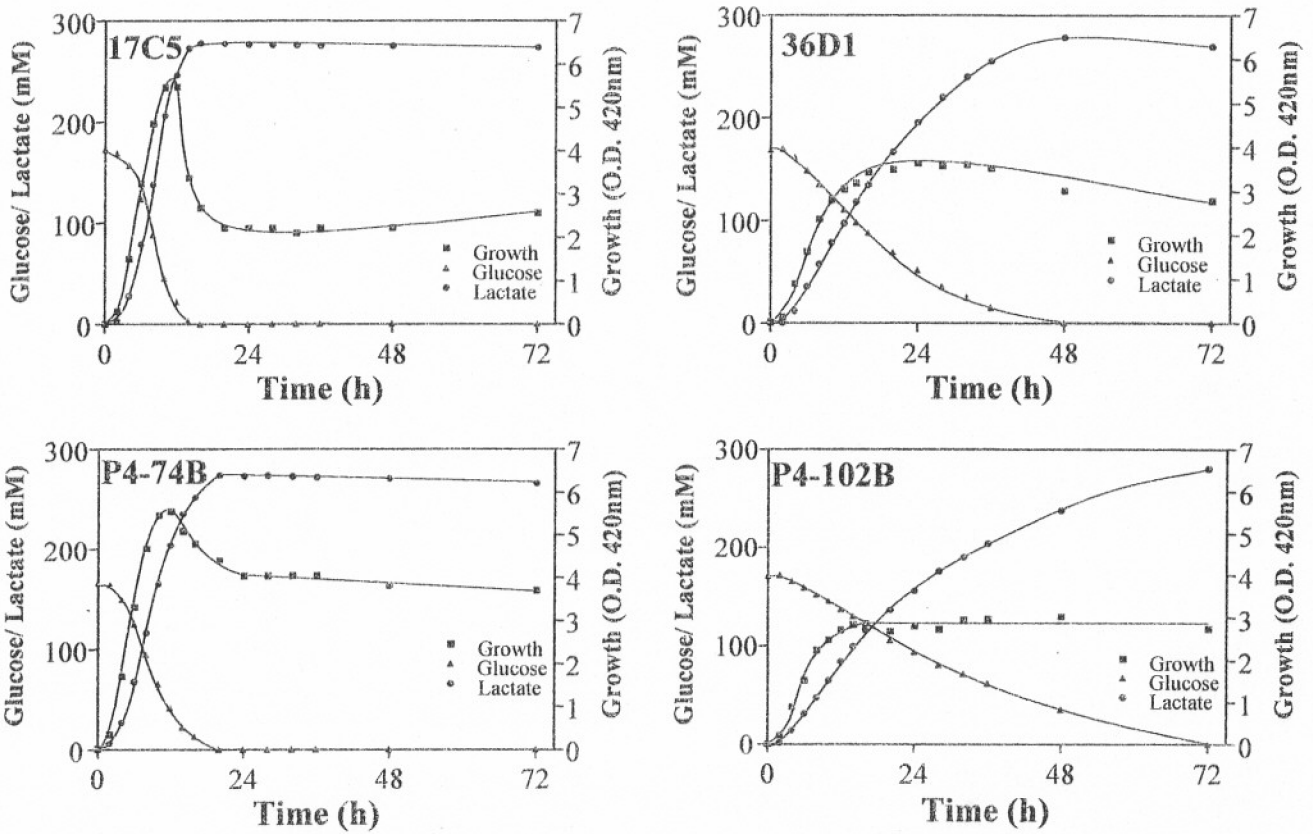

Figure 2. Glucose fermentation and lactic acid production by selected isolates in LB + glucose (3%) in a pH-stat at pH 5.0 and 50°C

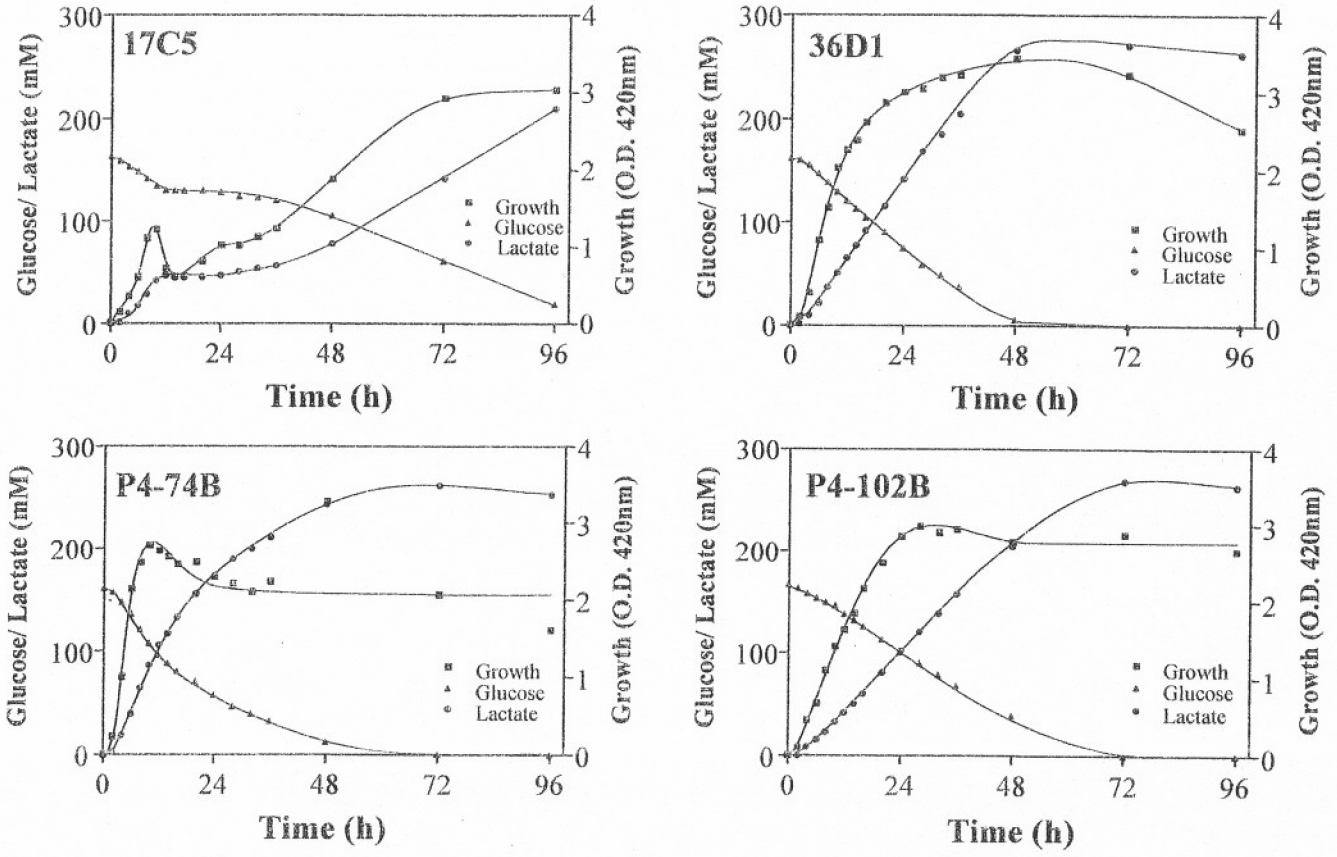

Figure 3. Glucose fermentation and lactic acid production by selected isolates in glucose (3%) - minimal medium with 1% corn steep liquor in a pH-stat at pH 5.0 and 50°C

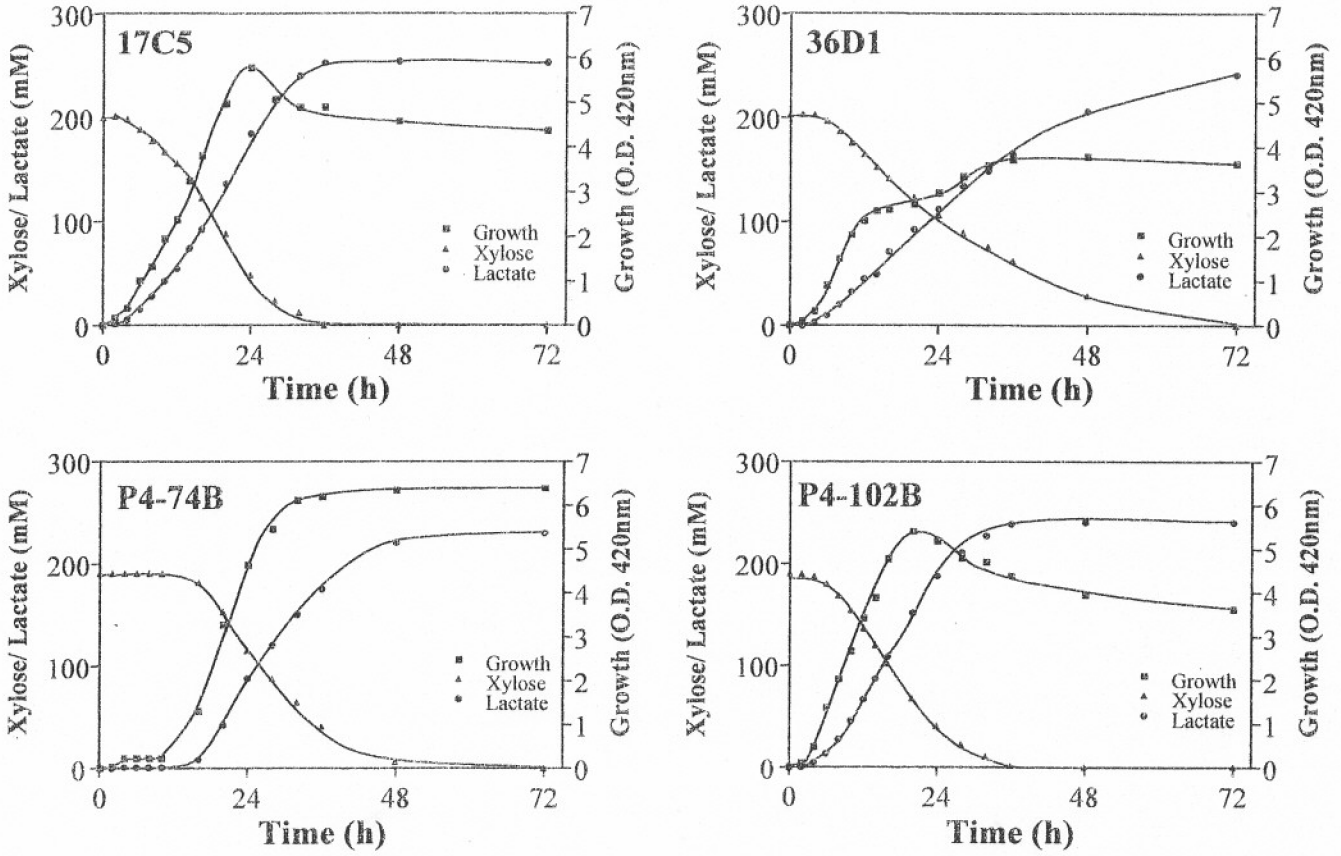

Figure 4. Xylose fermentation and lactic acid production by selected isolates in LB + xylose (3%) in a pH-stat at pH 5.0 and 50°C

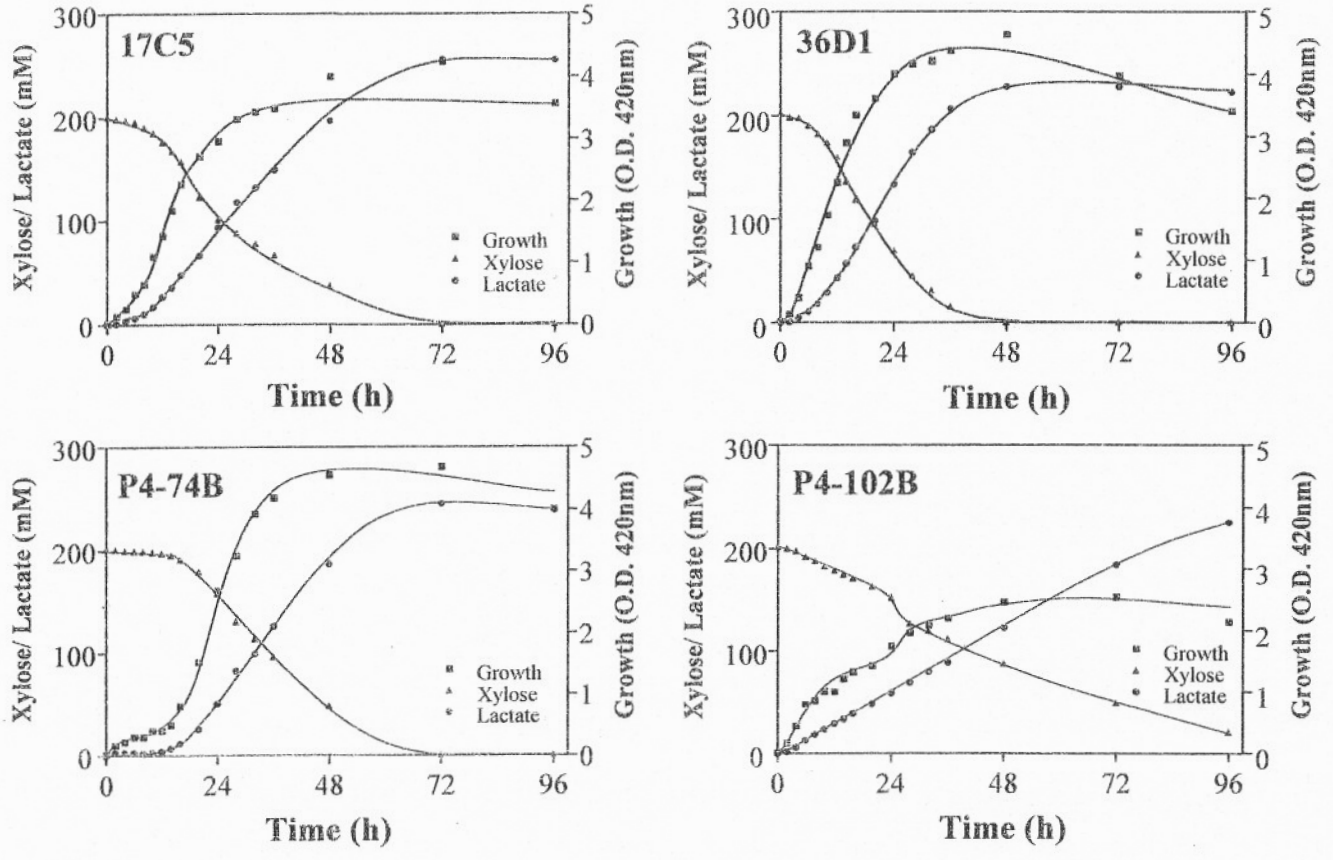

Figure 5. Xylose fermentation and lactic acid production by selected isolates in xylose (3%) - minimal medium with 1% corn steep liquor in a pH-stat at pH 5.0 and 50°C

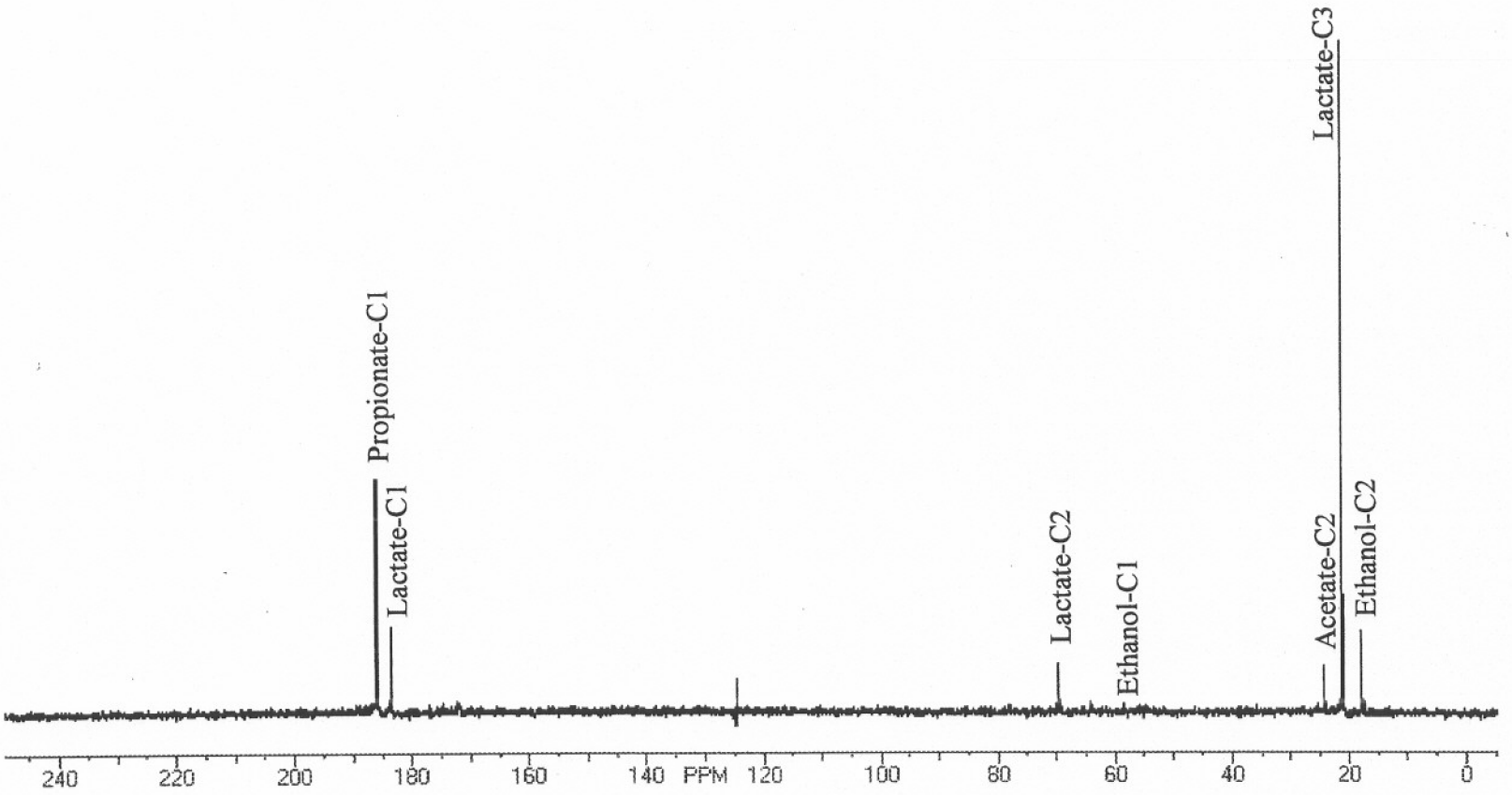

Fig. 6. ^{13}C-NMR spectrum of spent medium of strain 36D1 grown in LB+ $^{13}C_1$-Xylose (1.2%; 20.8% ^{13}C enrichment) at 50°C. pH of the culture was maintained between 6.0 and 7.0 by manual addition of 0.5 N KOH. $^{13}C_1$-propionic acid (50 mM) served as a standard.

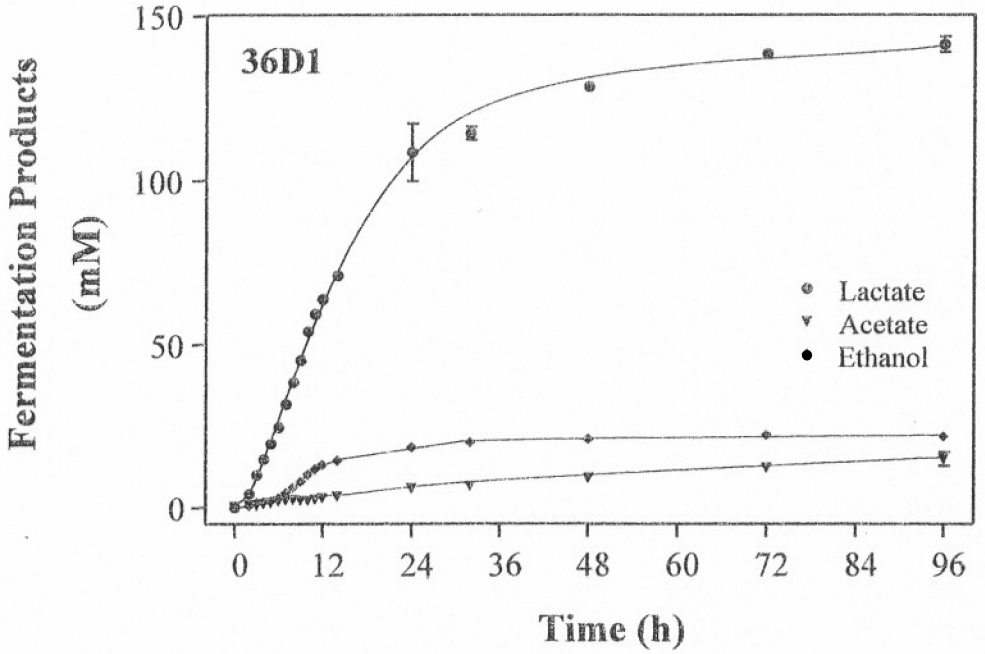

Figure 7. Simultaneous saccharification and fermentation (SSF) of Solka-Floc (2%) by strain 36D1 in the presence of 15 FPU Spezyme CE (Genencor)/g Solka-Floc in mineral salts medium with 1% corn steep liquor in a pH-stat at pH 5.0 and 50°C

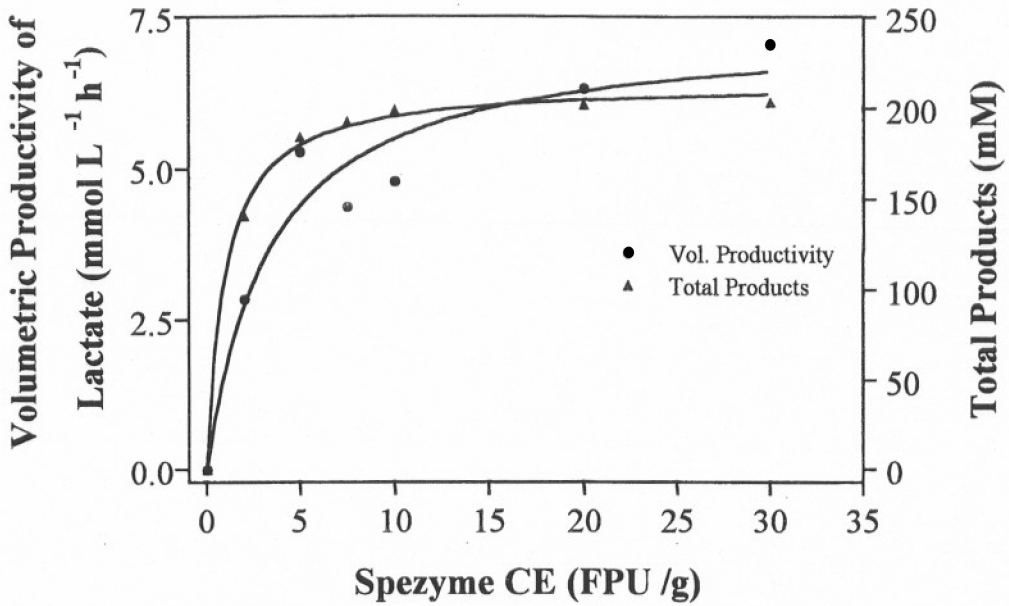

Figure 8. Effect of Spezyme CE concentration on SSF of crystalline cellulose, Solka-Floc, in mineral salts medium with 1% corn steep liquor in a pH-stat at pH 5.0 and 50°C, by strain 36D1. Total products represents lactate, acetate, ethanol and succinate.

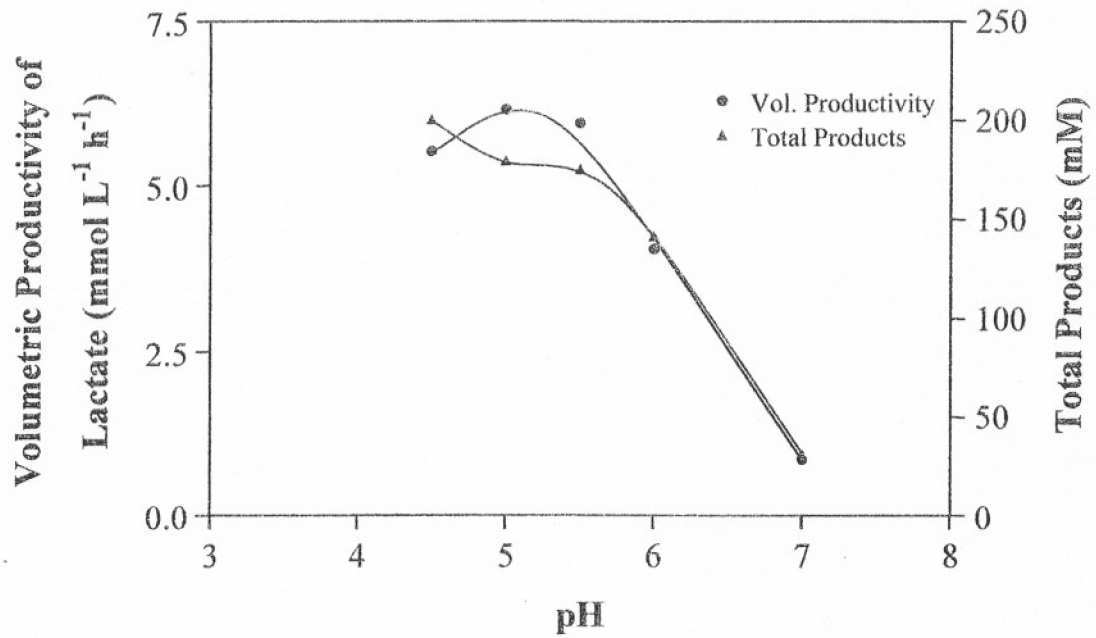

Figure 9. pH profile of SSF of Solka-Floc by strain 36D1with 15 FPU Spezyme CE /g Solka-Floc in mineral salts medium with 1% corn steep liquor in a pH-stat at 50°C. Total products represents lactate, acetate, ethanol and succinate.

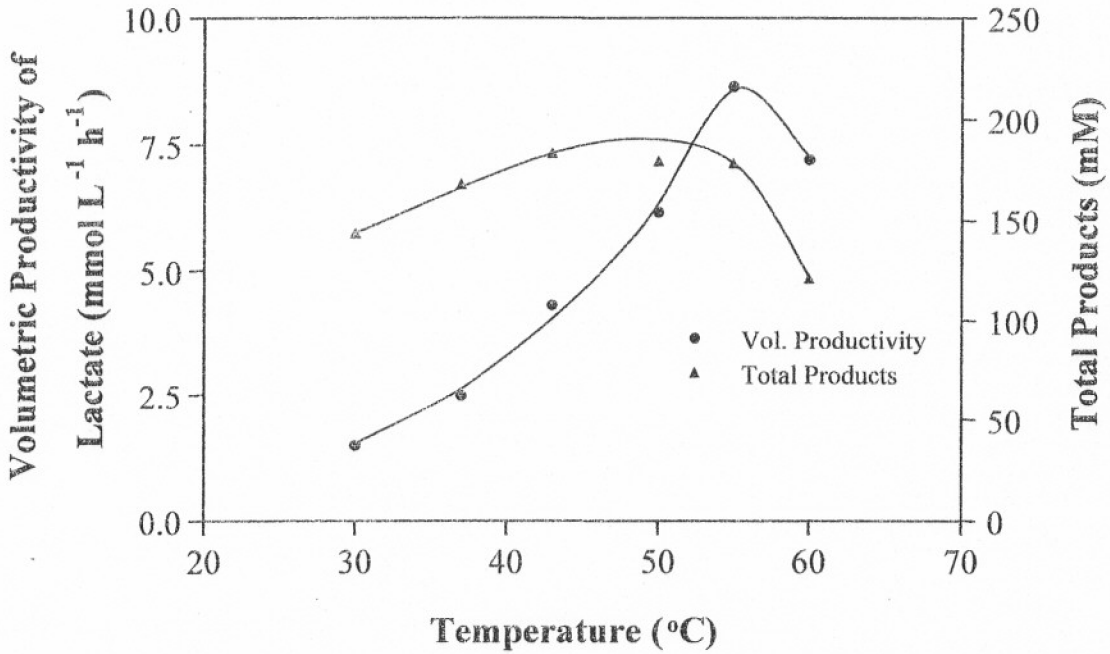

Figure 10. Temperature profile of SSF by strain 36D1 with 15 FPU Spezyme CE /g Solka-Floc in mineral salts medium with 1% corn steep liquor in a pH-stat at pH 5.0. Total products represents lactate acetate, ethanol and succinate.

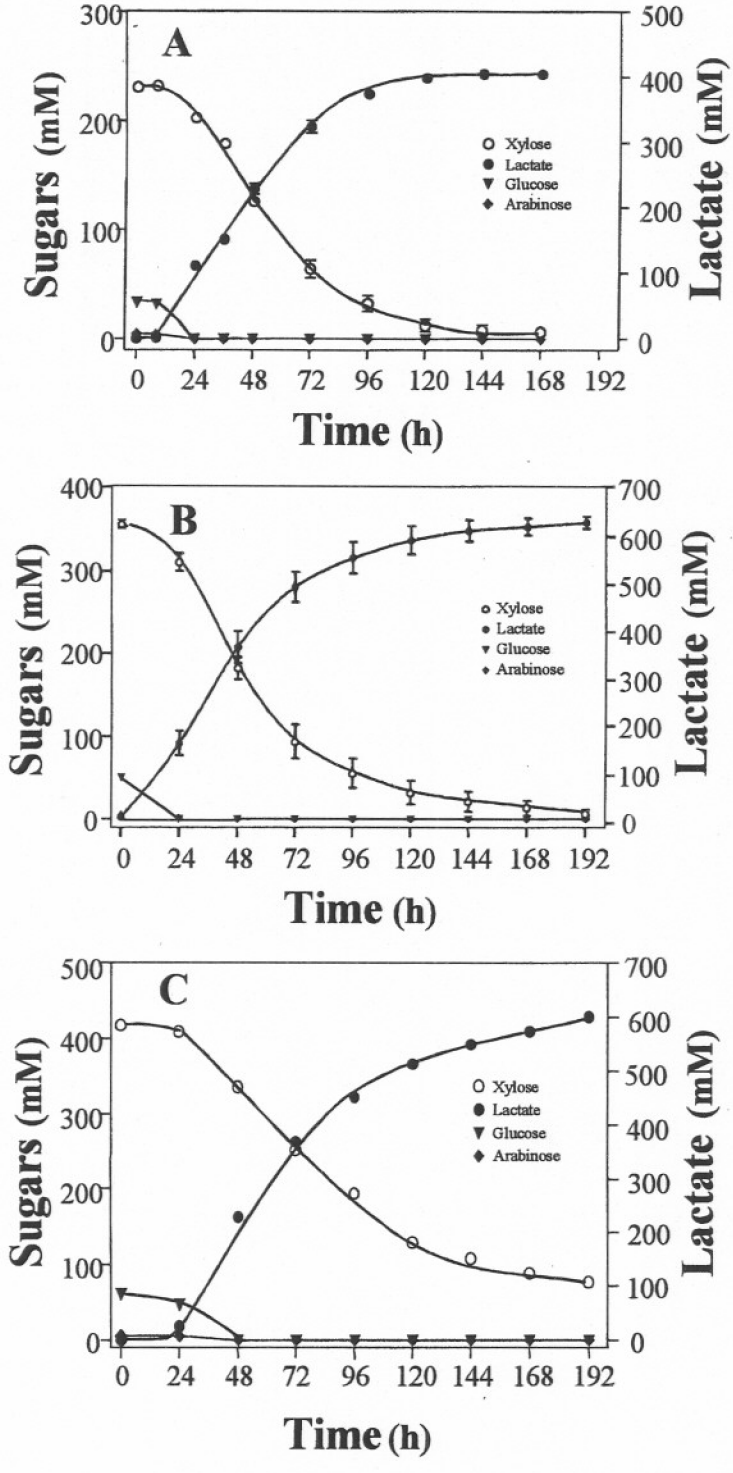

Figure 11. Fermentation of hemicellulose hydrolysate from sugar cane bagasse by *Bacillus* sp. strain 17C5 at pH 5.0 and 50°C. The initial total sugar concentrations were 256 mM (A), 412 mM (B) and 483 mM (C). In hydrolysate media, xylose represented 86% of the total sugars with glucose (12.5%) and arabinose (1.5%) as minor components. Lactate values have been corrected to reflect production by subtracting the small amount of lactate present in media (corn steep liquor) prior to inoculation.

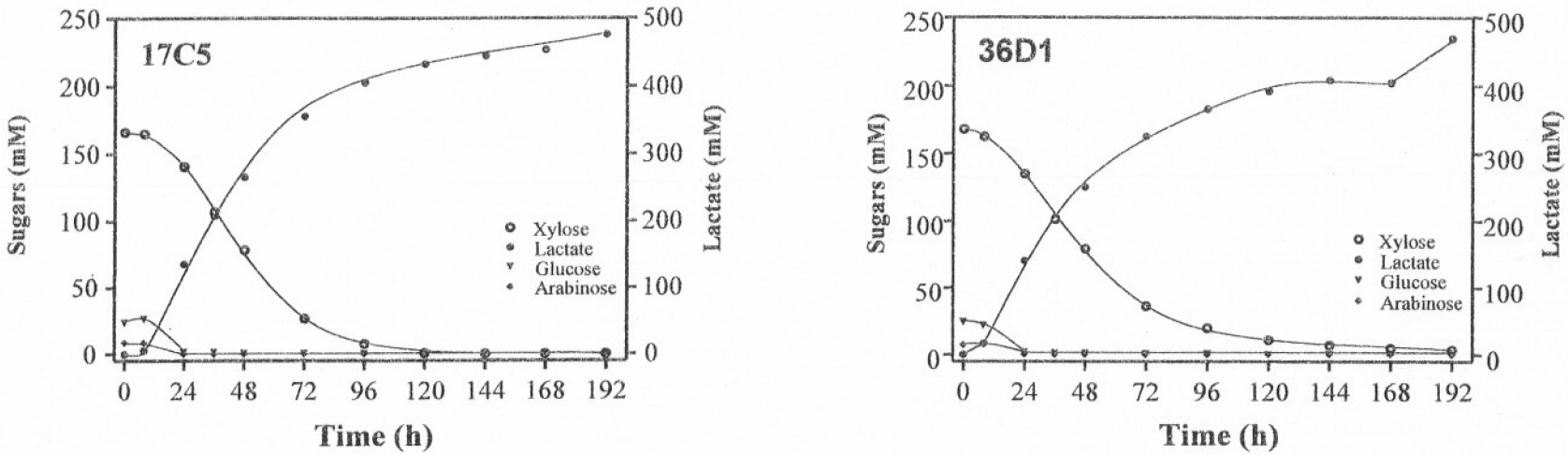

Figure 12. Simultaneous saccharification and co-fermentation (SSCF) of sugars present in overlimed sugarcane bagasse hemicellulose hydrolysate (40%) and Solka-Floc (2%) in the presence of 10FPU Spezyme CE/g Solka-Floc by strains 17C5 and 36D1 in mineral salts medium with 0.5% corn steep liquor in a pH-stat at pH 5.0 and 50°C

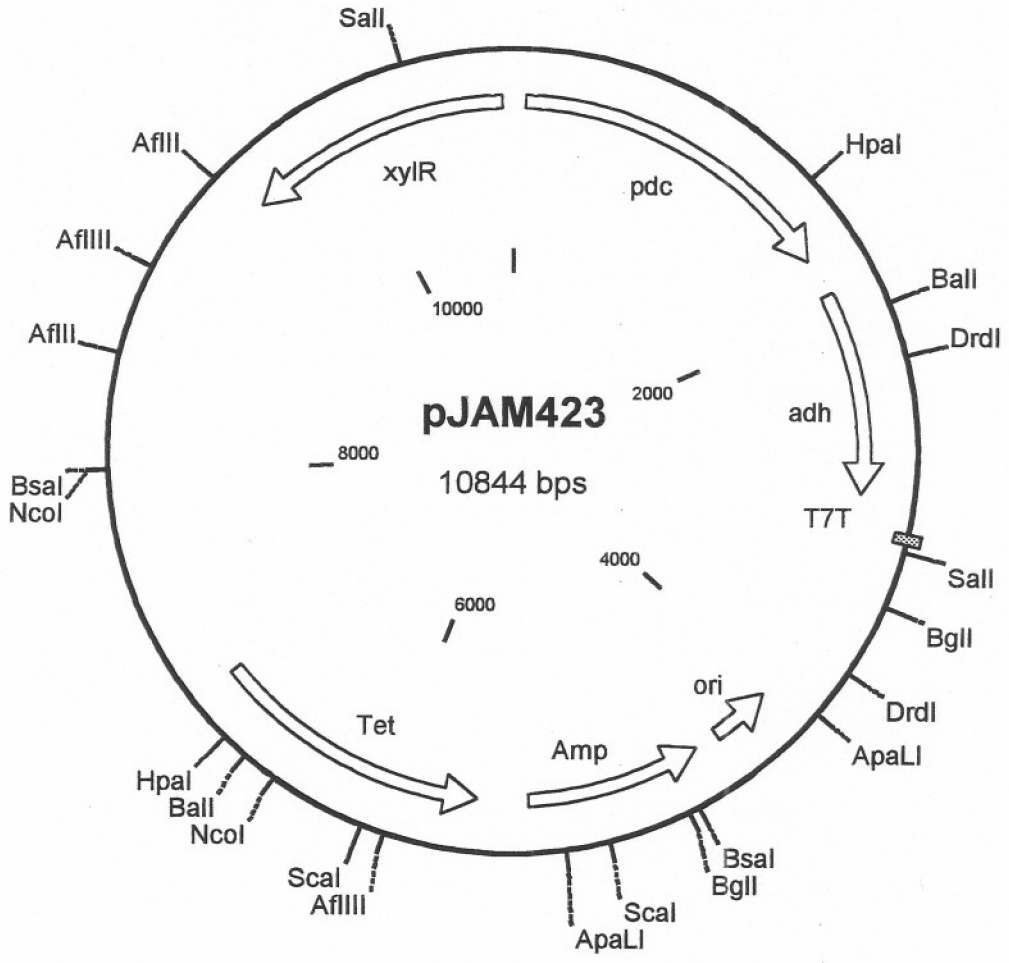

Fig. 13. Restriction map of plasmid pJAM423 containing *Sarcina ventriculi pdc* gene and *Geobacillus stearothermophilus adh* gene under the control of *xylA* promoter.

This plasmid can replicate in both *E. coli* and in Gram-positive bacteria such as *Bacillus megaterium.*

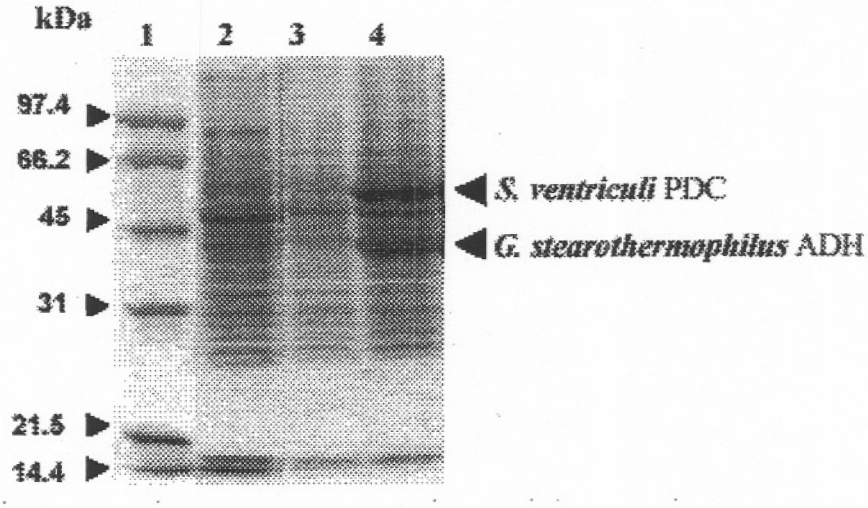

Fig. 14. Production of *S. ventriculi* pyruvate decarboxylase and *G. stearothermophilus* alcohol dehydrogenase from plasmid pJAM423 in *B. megaterium*.

The genes were induced with xylose and the proteins in the cell extracts were separated by reducing SDS-PAGE and stained with Coomassie blue R-250. Lane 1, Molecular mass standards; Lane 2, cell lysate (20 μg protein) of *B. megaterium* (pWH1520) with vector plasmid alone grown with xylose; Lanes 3 and 4, cell lysates (20 μg protein each) from *B. megaterium* (pJAM423) grown without and with xylose, respectively.