Sweet As Sugar-Efficient Conversion of Lactose into Sweet Sugars Using a Novel Whole-Cell Catalyst

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Sweet as Sugar - Efficient conversion of lactose into sweet sugars using a novel whole-cell-catalyst

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Abstract

Lactose, the sugar contained in milk, has a low sweetness. We have constructed an efficient whole-cell catalyst (WCC), that can be grown on dairy waste and which is able to convert lactose into a mixture of sugars as sweet as sucrose. The WCC is based on *Corynebacterium glutamicum* ATCC13032, which has been engineered to metabolize lactose, to express xylose and arabinose isomerase and to eliminate byproduct formation. When introduced in concentrated cheese whey permeate, its content of 98 g/L lactose was completely hydrolyzed and the liberated sugars partially isomerized into 23.5 g/L fructose and 20.4 g/L tagatose, which corresponds to a 49% conversion of the glucose and a 44% conversion of galactose. The latter is similar to what can be obtained using purified enzymes. The new technology enables better resource utilization and allows for dairy waste to be converted into a valuable food sweetener with many potential uses.

**Keywords:** whole-cell catalyst, *Corynebacterium glutamicum*, lactose hydrolysis, sugar isomerization, sweetener
**Introduction**

Lactose, a disaccharide consisting of one glucose molecule joined to a galactose molecule by a β-1, 4-glycosidic linkage, is the primary sugar found in the milk of mammals. Lactose first emerged as a commercial commodity during the 18th century \(^1\), and with the later expansion of the dairy industry increasing volumes of various lactose-containing (waste) streams followed. Whey and whey permeate are examples of such streams that are generated from manufacture of cheese and other fermented dairy products. The amount of lactose annually produced in the form of dried whey is estimated to be more than 1,400,000 tons \(^2\).

Whey permeate, or deproteinized whey, has several applications within the food and confectionery industry, where it promotes sweetness and adds flavor to the product \(^1\). Beyond food applications, lactose is also used by the pharmaceutical industry as a bulking agent \(^3\). With the explosive rise in cheese production since the latter half of the 20th century, an urgent need for new alternative ways to utilize whey has arisen. Two types of use have gained most attention, where the first is microbial conversion of lactose into different value added compounds \(^4,5\), and the second involves hydrolysis of lactose into glucose and galactose that can be used for sweetening purposes.

Lactose has a low sweetness (16% of the Perceived Sweetness of Sucrose, PSS) compared to its monosaccharide moieties, glucose (74% PSS) and galactose (32% PSS) \(^6\). Hydrolysis of lactose into the glucose/galactose syrup extends the use of whey permeate as a sweetening agent, not only because the syrup is three times sweeter than lactose \(^7\), but also because 70% of the world’s population suffer from lactose intolerance, due to an inability to metabolize lactose \(^8\).

Chemical hydrolysis of lactose requires harsh conditions such as very high temperatures (up to 150°C) and extremely acidic conditions (pH<1.5), which result in formation of undesirable byproducts \(^9\) and hydrolysis using enzymes is thus the preferred choice. Enzymatic hydrolysis of
lactose is a quite common procedure, and is typically carried out at temperatures between 30°C and 50°C. The purified beta-galactosidase are normally derived from various microbes, either natural or recombinant.

Although hydrolysis of lactose increases sweetness, the liberated glucose and galactose are not excessively sweet, and, in particular, galactose has a low sweetness. It is possible to isomerize glucose into fructose and galactose into tagatose by using enzymes, and thereby not only increase the overall sweetness but also reduce the glycemic index, which is desirable for food applications. As for lactose hydrolysis, isomerization using purified microbial enzymes is preferred before the use of chemical catalysts, as the conditions are milder and the process less sophisticated. Since the 1970s, high-fructose corn sirup (HFCS) has been used as a sweetener in foods and soft drinks and the market for HFCS is immense. Tagatose came on the market later, however, is also considered a promising sugar substitute due to its high-sweetness (92% to sucrose) and low-calorie features.

Whole-cell catalysis is not a novel approach, and in the past few decades, WCC have been used to produce a wide variety of products in various industries. Whole-cell catalysis potentially has several advantages over the use of purified enzymes. First, there is no need for a costly enzyme purification step. Second, the protected intracellular environment stabilizes enzymes under harsh conditions. Third, for re-use, whole cells can easily be recovered by simple filtration or the cells can be immobilized.

In this study, we modify *C. glutamicum*, a Generally Recognized As Safe (GRAS) Gram-positive bacterium to become a host for expressing the relevant enzymes needed for lactose hydrolysis and subsequent isomerization. The strain applied is able to ferment lactose, and lacks lactate dehydrogenase. We demonstrate that this engineered *C. glutamicum* strain efficiently can convert
whey permeate into a sweet syrup in a single-step process.

Methods

Growth medium and conditions.

*Escherichia coli* (*E. coli*) strains were grown aerobically in Luria-Bertani broth (LB) at 37°C, and *C. glutamicum* strains were cultivated in Brain Heart Infusion broth (BHI) or CGXII minimal medium at 30°C with 200-rpm shaking. When appropriate, kanamycin was added to a concentration of 50 μg/mL for *E. coli* and 25 μg/mL for *C. glutamicum*, and chloramphenicol was used at a concentration of 25 μg/mL for *E. coli* and 8 μg/mL for *C. glutamicum*. When needed, one mM of IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to induce gene expression.

Deletion of the *ldhA* gene in *C. glutamicum*.

Preparation of electrocompetent *C. glutamicum* cells as well as transformation were carried out as described previously. The deletion of the *ldhA* gene in JS95 was conducted via a two-step homologous recombination procedure using the vector pK18mobsacB. Briefly, two 800-bp downstream and upstream gBlock fragments of *ldhA* were ordered from Integrated DNA Technologies (Coralville, IA, U.S.) and cloned into the multiple cloning site of pK18mobsacB with the Gibson cloning kit (SGI-DNA, La Jolla, CA, U.S.). The resulting plasmids were transformed into the electrocompetent JS95, and clones with the plasmid successfully integrated were selected on BHI plates with kanamycin. Counter-selection was performed on BHI with 10% sucrose. Successful *ldhA* knockouts were confirmed by Sanger sequencing, and strain JS99 is one of these.

Plasmid-based expression of xylose isomerase and arabinose isomerase (construct A).

The *xylA* gene from *Arthrobacter* sp. NRRL B3728 encoding xylose isomerase and the *araA* gene from *Bacillus coagulans* NL01 encoding arabinose isomerase were chosen for the purpose of
isomerizing glucose into fructose and galactose into tagatose respectively. Two gBlock gene
fragments were ordered from Integrated DNA Technologies (Coralville, IA, U.S.) and assembled
with the XbaI digested vector pEC-XC99E. The ligation mix was first electroporated into the E.
coli TOP10 cells. Colonies with correct construct (construct A, Figure S1) were verified by colony
PCR and followed by Sanger sequencing. The plasmids were subsequently isolated from E. coli
using the Zyppy Plasmid Miniprep Kit (Irvine, CA, U.S.) and electroporated into the C. glutamicum
competent cells.

Plasmid-based expression of xylose isomerase and arabinose isomerase using tandem
repetitive promoters (construct B)

The shuttle plasmid pEC-XC99E for C. glutamicum was used as the backbone for the construction
of the new expression vector. First, two gBlock gene fragments (Integrated DNA Technologies,
Coralville, IA, U.S.), each of which contained a trc promoter sequence, were assembled into the
BamHI site of pEC-XC99E using the Gibson cloning kit (SGI-DNA, La Jolla, CA, U.S.) resulting
in the plasmid pJS150. Afterwards, two additional trc promoter sequences were introduced into the
SalI site of pJS150 with the same cloning strategy and generated a new SalI site at the 3’ of the
promoter sequence. The final vector, pJS151, contained four additional trc promoters downstream
of the original promoter in pEC-XC99E. The xylA and araA genes were cloned into the new SalI
site of pJS151 to generate the construct B.

Construction of the expression plasmid with lacZ (construct C).

The lacZ gene was PCR amplified from the genomic DNA of Streptococcus thermophilus (S.
thermophilus) with the following primers placZ-F:
ACCTCCTGGGCTCCCGCTAATCGACCTGCAGTTATTACCTTCAAAAAAGG and placZ-
R:CATCCGCCAAAAACAGCCAAGCTTGACATGCCTTCAATTTAGTGGTCTCAATCA. The
fragment was cloned into the *pstI* site downstream the *xylA* and *araA* gene in the construct B with the Gibson cloning (SGI-DNA, La Jolla, CA, U.S.) to generate construct C.

**Sugar hydrolysis and isomerization.**

One mL of overnight culture of the cell catalyst in BHI medium was inoculated in 50-ml CGXII supplied with 5% lactose and 1mM IPTG. After 24 hours of cultivation, the cells were harvested by centrifugation (5,000 g for 10 minutes). The pellet was washed once with 0.9 % NaCl solution. For the hydrolysis of lactose and further isomerization, 15 g/L DCW (dry cell weight) cells were resuspended in 40 mM MOPS solution (pH = 7.5) containing lactose, and incubated under static conditions at 60°C. For the hydrolysis of the whey lactose, the pH of ultra-filtrated cheese whey was adjusted to pH 7.5 prior to the use for the whole-cell catalysis. The concentration of cells and other reaction settings used in whey was same as that used in lactose MOPS solution.

**Thermal stability test for β-galactosidase, xylose isomerase and tagatose isomerase.**

The thermal stability of β-galactosidase and isomerases was monitored after incubation of the WCC in whey permeate under static conditions at 60°C. The WCC was sampled at periodic intervals for the measurement of the residual activity in 40 mM MOPS solution (pH = 7.5) with either 50 g/L lactose, glucose or galactose. The activities at 0 hour were defined as 100%.

**HPLC analysis of sugars and acids.**

The concentration of lactate was determined on an Ultimate 3000 high-performance liquid chromatography system (Dionex) equipped with an Aminex HPX-87H column (Bio-Rad) and a Shodex RI-101 detector (Showa Denko K.K.). The column oven temperature was set to 60°C and the mobile phase consisted of 5 mM H$_2$SO$_4$. The flow rate used was 0.5 mL/min. For the
measurement of lactose, glucose, galactose, fructose and tagatose (Sigma-Aldrich), the same system
and setup were used, except for the column (Agilent Hi-Plex Ca column) and mobile phase (water).

Results & Discussion

*C. glutamicum* retains a low glycolytic activity at 60°C

Previously we have engineered *C. glutamicum* into efficiently metabolizing lactose, by introducing
the lactose permease and the β-galactosidase from *Streptococcus thermophilus* and the Leloir
pathway from *Lactococcus lactis* on the chromosome. *C. glutamicum* readily grows under aerated
conditions, but not when deprived of oxygen. We hypothesized that the engineered strain, JS95,
when incubated at elevated temperatures could serve as a whole-cell catalyst for hydrolyzing
lactose due to heat-induced permeabilization of its membrane, and tested this. Using 15 g/L DCW
cells, 114.20 g/L lactose could be completely hydrolyzed within 24 hours at 60°C, and a total of
108.13 g/L glucose and galactose were generated (Table 1). The sugar recovery rate reached
94.68%. Besides the glucose and galactose, we found that lactate had been formed in small amounts
(3.05 g/L). *C. glutamicum* is an aerobic bacterium that ceases to grow under anaerobic conditions,
however, it retains the ability to metabolize sugars into organic acids, mainly lactate, due to an
active glycolysis and lactate dehydrogenase. We expected that the high temperature incubation
would inactivate central metabolism in the mesophilic *C. glutamicum*, however, lactate formation
indicated that this was not the case. Previously it has been reported for some mesophiles, with a
high abundance of glycolytic enzymes, that some residual activity persists for a period after high
temperature exposure. Howell *et al.* demonstrated that most glycolytic enzymes in mesophilic
bacteria still maintained 50% activity after a half-hour heat treatment at 55°C - 70°C. To
eliminate lactate formation, we decided to delete the *ldhA* gene, which is solely responsible for the
formation of lactate in *C. glutamicum*. Indeed after deleting *ldhA*, lactate formation ceased, and
the sugar recovery rate increased to 97.21% compared to 94.68% with the reference strain JS95 (Table 1). The \textit{ldhA}-null strain JS99 appeared to be an ideal starting point for further engineering.

**Concurrent hydrolysis of lactose and isomerization of its constituent monosaccharides by using recombinant \textit{C. glutamicum}-based cell catalysts.**

To isomerize glucose and galactose into fructose and tagatose respectively, we decided to rely on the promiscuous activities of xylose isomerase (fructose isomerase) and arabinose isomerase (tagatose isomerase). First, we introduced \textit{xylA} gene from \textit{Arthrobacter} sp. NRRL B3728 \textsuperscript{25} and \textit{araA} gene from \textit{Bacillus coagulans} strain NL01 \textsuperscript{26} in strain JS99, by using the plasmid pEC-XC99E as expression vector (construct A), and obtained strain JS154. After a 72-h static incubation with 15 g/L DCW cells of JS154 at 60°C, 95 g/L lactose could be hydrolyzed and converted into a mixture of 23.0 ± 0.5 g/L glucose, 22.8 ± 0.2 g/L fructose, 32.7 ± 0.9 g/L galactose, and 12.8 ± 0.4 g/L tagatose. In this process, the high temperature exposure created a leaky cell envelope, through which the substrate could enter and the products could leave. The conversion was efficient and only small amounts of lactose remained (0.8 ± 0.0 g/L). The amount of glucose and galactose converted into fructose and tagatose were 50% and 28% respectively. In a comparable study \textsuperscript{26}, where the \textit{araA} gene from \textit{Bacillus coagulans} strain NL01 was expressed in \textit{E. coli}, the amount of galactose converted into tagatose reached a maximum of 40% when applying whole-cell catalysis with galactose as the substrate. The lower sugar isomerization efficiency achieved in our setting indicated that there was room for improvement. It is possible that the \textit{araA} gene was expressed less well in \textit{C. glutamicum} than in \textit{E. coli}, e.g. because of differences in transcription/translation efficiency or because of differences in copy-number of the plasmid used for expressing \textit{araA}. For \textit{E. coli}, high copy-number expression vectors and strong inducible promoters are widely available, but this is hardly the case for \textit{C. glutamicum} \textsuperscript{33}. Second, the fact that we had integrated the β-galactosidase in the chromosome, could have generated a bottleneck for subsequent isomerization.
It is possible that lactose hydrolysis was a rate-limiting step which potentially could exacerbate the effect due to loss of activity of the xylose isomerase and arabinose isomerase at high temperatures. For all of these reasons, we attempted to improve the expression of the three genes.

**Optimization of isomerization efficiency by increasing gene expression.**

In order for whole-cell catalysts to be efficient, it is essential that the relevant enzymes are expressed to a sufficiently high level. Most of the expression vectors for *C. glutamicum* rely on the tac/trc promoters, and although these promoters are derived from *E. coli*, they provide inducible high-level expression of target genes in *C. glutamicum*. However, the copy number of *C. glutamicum* vectors is generally low (~20 copies per cell), and this could be a limiting factor for overall expression. Shin *et al.* compared the influence of different media on the level of heterologous protein expression in *C. glutamicum*, and found that Riesenberg broth supported the highest expression levels when compared to other commonly used media such as BHI and CGXII. However, the use of a specific medium could restrict industrial applications, as industrial media often contain many complex components. When we used construct A, the conversion of galactose into tagatose was merely 12% lower than that obtained in the previous study using *E. coli*. To avoid the risk of overloading cellular metabolism by using high copy number expression vectors, we decided to introduce multiple trc promoters upstream of the genes in the vector pEC-XC99E, which appeared to be a fast and controllable manner in which the expression could be increased.

Four additional trc promoters were introduced and in the new construct (B), the *xylA* and *araA* genes were transcribed from five tandem trc promoters (Figure S1). The strain JS99 with construct B was designated JS155. When the isomerization was performed with JS155, an increase in productivity of approximately 60% and 120% for fructose and tagatose respectively was observed (Figure 1& 2B), and the tagatose titer increased from 12.8 g/L to 19.4 g/L. With the new construct
B, 42% of the released galactose was converted into tagatose (Figure 2A), which clearly demonstrates that the expression level was the limiting factor. The final fructose titer did not change, however, this was not expected as a 50% conversion corresponds to the equilibrium between galactose and tagatose at 60°C \(^{12}\). Therefore, with a higher \textit{xylA} expression, only the maximum productivity could be improved, and not the conversion ratio. The xylose isomerase used in this study is a thermally stable enzyme \(^{12}\), and the partial thermal denaturation taking place during the isomerization process (72h at 60°C) apparently was not significant as equilibrium could be reached. This was clearly not the case for arabinose isomerase, where complete loss of activity was observed before equilibrium could be reached (Figure 2).

Only one copy of the \textit{lacZ} gene was present in the chromosome of strain JS99, in contrast to the \textit{xylA} and \textit{araA} genes, that both were expressed on plasmids from strong promoters. The relatively low intracellular level of \(\beta\)-galactosidase probably limited the maximum productivity of fructose and tagatose, due to a slow release of glucose and galactose. To overcome this potential limitation, we made a new construct C, by inserting an additional \textit{lacZ} gene downstream of the \textit{xylA} and \textit{araA} genes in construct B (Figure S1). Strain JS99 carrying construct C was designated JS156. This final catalyst was able to completely hydrolyze lactose within 12 hours, as compared to 24 hours for the catalyst based on construct B (Figure 3) (the construct B). With construct C, both the maximum production rates of fructose and tagatose were further improved by 45% and 50% compared to construct B on lactose respectively. As expected, the increased cleavage rate of lactose did not improve the conversion into fructose and tagatose, as the equilibrium between glucose/fructose and galactose/tagatose at 60°C had been reached \(^{12,26}\).

**Simultaneous whey lactose hydrolysis and isomerization of glucose and galactose**

Eventually, we applied the cell catalyst JS156 on concentrated cheese whey permeate. The cheese
whey ultra-filtrated permeates contained approximately 98 g/L lactose. With the incubation of 15 g/L DCW JS156 cells at 60°C for 48 hours, the final whey-based GGFT syrup was composed of 24.3 ± 0.0 g/L glucose, 25.7 ± 0.1 g/L galactose, 23.5 ± 0.3 g/L fructose and 20.4 ± 0.1 g/L tagatose. A high thermostability is a desired property for a catalyst to be applied at elevated temperatures, and we therefore examined the stability of the three enzyme activities over time at 60°C. It was found (Figure S2) that the activity of the two isomerases remained high and unaffected by the high temperature exposure for more than 48 hours. The β-galactosidase activity, however, started to reduce after 12 hours, but prior to this, the lactose had been completely hydrolyzed (Figure 3).

In recent years, tagatose, as a GRAS food additive, has been extensively researched as a functional sweetener. Several researches on tagatose production from lactose or lactose-containing feedstock have been reported. Xu et al. reported a single-step process for tagatose production from lactose based on recombinant E. coli whole cells expressing an arabinose isomerase from Lactobacillus fermentum CGMCC2921 and a β-galactosidase from Thermus thermophiles HB27, where a maximum yield of 20.2% tagatose could be reached. To improve the productivity and yield, cell permeabilization and immobilization usually is carried out. Jayamuthunagai et al. produced tagatose from lactose in whey permeate by using a combination of enzyme treatment (beta-galactosidase) and alginate immobilized Lactobacillus plantarum cells (arabinose isomerase), and achieved a conversion of 38%, but the research only focused on conversion of the galactose moiety of lactose without considering to the possibility of generating sweetness from glucose. Rhimi et al. have previously reported that it is possible to produce tagatose and fructose from a mixture of galactose and glucose using an E. coli WCC expressing arabinose isomerase and xylose isomerase. Rhimi et al. used a mixture of glucose and galactose in MOPS buffer and a WCC that had been grown in LB medium, a setup that is quite far from real-life applications. Even though Torres et al. reported the
use of an immobilized tri-enzymatic system composed of β-galactosidase, xylose isomerase and arabinose isomerase for bioconversion of lactose, simultaneous hydrolysis of lactose and isomerization of the released sugars using a whole cell catalyst has not been reported. Torres et al. found that lactose hydrolysis was incomplete in all their experiments employing tri-enzymatic system because of product inhibition by galactose and glucose\textsuperscript{40}. When we employed our whole-cell catalyst in whey permeate, not only did we achieve complete lactose hydrolysis, but also efficient isomerization of glucose and galactose. There was no residual lactose, a 49\% conversion of the glucose into fructose and a 44\% conversion of the galactose into tagatose, which is comparable to what can be achieve using purified enzymes.

In a one-step process, the sweetness of whey was increased by 440\% and 160\% compared to the untreated whey and whey that merely had been hydrolyzed into glucose and galactose, respectively (Figure 4). The conversion was associated with a decrease in glycemic index by 25\%, when compared to untreated whey (Figure 4). The results clearly show that microbial catalysis is an efficient approach for turning cheese whey into a value-added sweetener, with potential benefits to human health.

In conclusion, we have presented a novel approach for valorizing cheese whey permeate, a low- or negative value side stream generated in enormous amounts by the dairy industry. By co-expressing β-galactosidase, xylose isomerase and arabinose isomerase in \textit{C. glutamicum}, a cell catalyst was made that efficiently could convert whey lactose into a sweet GGFT syrup, with lower calorie content and glycemic index. It is well-established that a high sugar consumption can cause various lifestyle-diseases such as obesity and type 2 diabetes \textsuperscript{41} and there have been several initiatives from organizations (FAO/WHO) and health authorities aiming at lowering the consumption of sugar. The technology developed addresses these societal challenges, while at the same time supporting a better resource utilization at dairies. The generation of the \textit{C. glutamicum} cell catalyst is also cost-
effective, since we previously have demonstrated that the host strain can grow on whey permeate without addition of expensive nutrients. We believe that it is possible to optimize the performance of the cell catalyst, e.g. by making the cells more permeable. To increase stability and to facilitate reuse, immobilization approaches could be tested.

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Notes

The authors declare no competing financial interest.

Supporting Information Statement
The Supporting Information is available free of charge on the ACS Publications website.

Figure S1. The plasmid maps of different constructs used in the study. The \textit{xylA} gene sequence. The \textit{araA} gene sequence. Two gBlocks containing trc promoter sequence.

References


Figure captions

Figure 1. Comparison of fructose and tagatose formation from lactose using JS154 and JS155.
JS154: JS99 harboring construct A, where the isomerase genes (xylA, araA) are expressed from the
tac promoter in pEC-XC99E; JS155: JS99 harboring construct B, where the isomerase genes are
expressed from multiple tandem promoters inserted upstream the tac promoter in pEC-XC99E. The
schematic of the plasmids used can be seen in Figure S1.

Figure 2. Comparison of productivity and conversion ratio for different cell catalysts. A, fraction of
 glucose and galactose converted into fructose and tagatose; B, the maximum productivity of
 fructose and tagatose during the one-step lactose conversion. JS154: JS99 harboring construct A,
 where xylA and araB are co-expressed on pEC-XC99E; JS155: JS99 harboring construct B, where
 xylA and araB are co-expressed on pEC-XC99E from four additional trc promoters; JS156: JS99
 harboring construct C, where xylA, araB and lacZ were co-expressed on pEC-XC99E from four
 additional trc promoters (plasmid maps can be seen in Figure S1).

Figure 3. Comparison of lactose hydrolysis rate with JS155 and JS156. JS155: only one copy of
 lacZ on the chromosome of JS99. JS156: additional lacZ were co-expressed on pEC-XC99E from
 four additional trc promoters in JS99 (The illustration of plasmid maps can be seen in Figure S1).

Figure 4. Comparison of relative sweetness and glycemic index between raw whey and enzyme-
treated whey. Lactose-hydrolyzed whey, treated with JS99; Sugar-isomerized whey, treated with
JS156 which is JS99 bearing the construct C.
Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glu + Gal (g/L)</th>
<th>Lactate (g/L)</th>
<th>Sugar Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>JS95</td>
<td>108.13 ± 0.59</td>
<td>3.05 ± 0.06</td>
<td>94.68</td>
</tr>
<tr>
<td>JS99</td>
<td>111.01 ± 0.07</td>
<td>0 ± 0</td>
<td>97.21</td>
</tr>
</tbody>
</table>

Table 1. The effect of inactivating lactate dehydrogenase in the whole-cell catalyst JS95 on sugar recovery and lactate formation. JS99: the ldh deficient derivative of JS95.

Figure 1

Figure 2
Figure 3

Figure 4