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Research Article

**Metabolic characterization and transformation of the non-dairy *Lactococcus lactis* strain KF147, for production of ethanol from xylose**

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**Keywords:** ethanol production; *Lactococcus lactis* KF147; The phosphoketolase pathway; The pentose phosphate pathway; Xylose metabolism

**Abbreviations:** **LAB**, lactic acid bacteria; **CDW**, cell dry weight; **SALM**, the synthetic amino-acid medium with lipoic acid and MOPS; **J<sub>pk</sub>**, the phosphoketolase flux; **J<sub>pp</sub>**, the flux through the pentose phosphate pathway;

Accepted Article

**Abstract**

The non-dairy lactic acid bacterium *Lactococcus lactis* KF147 can utilize xylose as the sole energy source. To assess whether KF147 could serve as a platform organism for converting second generation sugars into useful chemicals, we characterized growth and product formation for KF147 when grown on xylose. In a defined medium KF147 was found to co-metabolize xylose and arginine, resulting in biphasic growth. Especially at low xylose concentrations, arginine significantly improved growth rate. To facilitate further studies of the xylose metabolism, we eliminated arginine catabolism by deleting the *arcA* gene encoding the arginine deiminase. The fermentation product profile suggested two routes for xylose degradation, the phosphoketolase pathway and the pentose phosphate pathway. Inactivation of the phosphoketolase pathway redirected the entire flux through the pentose phosphate pathway whereas over-expression of phosphoketolase increased the flux through the phosphoketolase pathway. In general, significant amounts of the mixed-acid products, including lactate, formate, acetate and ethanol, were formed irrespective of xylose concentrations. To demonstrate the potential of KF147 for converting xylose into useful chemicals we chose to redirect metabolism towards ethanol production. A synthetic promoter library was used to drive the expression of codon-optimized versions of the *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase, and the outcome was a strain producing ethanol as the sole fermentation product with a high yield corresponding to 83% of the theoretical maximum. The results clearly indicate the great potential of using the more metabolically diverse non-dairy *L. lactis* strains for bio-production based on xylose containing feedstocks.

## 1 Introduction

Second generation biorefineries rely on plant biomass not intended for human consumption as feedstock for producing various useful chemicals and products, and increasingly the chemical machinery of microbes is being harnessed for these transformations. One major challenge involves finding suitable microbes that can convert the sugars contained in the plant biomass with high efficiency and yield [1], and that have a high tolerance towards various growth inhibitors generated in the course of biomass pre-treatment.

Potential candidates could possibly be found within the group of Lactic Acid Bacteria (LAB) that generally are quite robust, e.g. many of its representatives can withstand relatively high concentrations of organic acids, alcohols and also of the inhibitors formed during pre-treatment [2, 3]. They can metabolize a far more comprehensive range of sugars than previously assumed, which adds to their favour [4], are able to grow at a relatively low pH and some even at quite high temperatures [5, 6]. The latter two properties could help reduce problems associated with contamination in industrial fermentations and thereby contribute to reducing operational costs. Finally there are many examples in the literature demonstrating the successful use of LAB as efficient cell factories [5–8].

*Lactococcus lactis* is mainly known for its role in cheese production, and it is considered as the model organism of LAB. Several of the *L. lactis* dairy strains have been thoroughly characterized and are typically quite fastidious and lack the ability to metabolize pentoses [9, 10], but *L. lactis* strains and many other LAB, isolated from plant niches are able to do this, and can metabolize a broad range of other sugars quite efficiently as well [3, 11–13]. In addition to their broader substrate range, most of these strains have fewer nutritional requirements. *L. lactis* normally has a fermentative metabolism, producing lactate when growing on rapidly fermentable sugars, e.g. glucose, and a mixture of acids and ethanol when growing on more slowly fermentable sugars [13]. There have been some conflicting reports in the literature concerning how pentoses are metabolized in LAB. Kandler et al. concluded that all LAB metabolize xylose only through the phosphoketolase pathway, producing equimolar amounts of lactate and acetate [13], while Tanaka et al. found that *L. lactis* IO-1 could metabolize xylose through two pathways: the non-oxidative pentose phosphate/glycolytic pathway and the

phosphoketolase pathway [14]. *L. lactis* lacks a transaldolase [15–17] and it appears that the pentose phosphate pathway operates in a different way, where the glycolytic enzymes phosphofructokinase and aldolase take part (Fig. 1) [18, 19].

In this study we have examined the non-dairy *L. lactis* strain KF147, which was originally isolated from mung bean sprouts [20]. We show, by carefully investigating product fluxes, that both the pentose phosphate pathway and the phosphoketolase pathway are involved in xylose degradation, and that the extent to which either pathway is used depends on xylose concentration. We furthermore delete or over-express various genes and study how flux distributions and product formation change. Finally we manage to redirect metabolism into ethanol production from xylose by expressing heterologous pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis*, thus clearly demonstrating the potential of KF147 as a platform for producing value-added compounds from second-generation feedstocks.

## 2 Materials and methods

### 2.1 Strains and plasmids

*L. lactis* subsp. *lactis* KF147 [20], *L. lactis* subsp. *lactis* IO-1 [14] and *L. lactis* subsp. *cremoris* MG1363 [16] were used in this study. For site-specific integration in the chromosomal attachment site (*attB*), the plasmid pLB65 was used for expressing the TP901-1 integrase [21]. For cloning purposes *Escherichia coli* ABLE-C (Stratagene) or MC1000 [22, 30] was used. The plasmid pG<sup>+</sup>host8 was used for deletion of genes and pG<sup>+</sup>host4 was used for inserting *attB* [23]. The plasmid pLB85 [21] was used for site-specific integration of the *ptk* gene into the chromosomal *attB* site. All strains and plasmids used in this study are listed in Table S1.

### 2.2 Growth media and growth conditions

*E. coli* strains were grown with aeration at 28°C in modified LB medium (10 g L<sup>-1</sup> peptone from casein, 5 g L<sup>-1</sup> yeast extract, 4 g L<sup>-1</sup> NaCl). For determination of xylose and product fluxes, *L. lactis* was grown in batch cultures at 30°C in defined SA medium [24] modified by substituting acetate with 2 µg ml<sup>-1</sup> lipoic acid and increasing MOPS to a final concentration of 120 mM (SALM). Xylose was added to

concentrations of 0.2%, 1.0% or 3.0% (w/v). To keep the cultures homogenous, slow stirring with magnets was used. For growth experiments in rich medium, M17 [25] was supplemented with xylose to concentrations of 1.0%, 3.0%, 5.0%, 7.0% and 10.0%. The cultures were incubated both at 30°C and 37°C. For other purposes, M17 was supplemented with 1% (w/v) glucose. When appropriate, antibiotics were added to the following concentrations: tetracycline, 8  $\mu\text{g ml}^{-1}$  for *E. coli* and 5  $\mu\text{g ml}^{-1}$  for *L. lactis*; erythromycin, 150  $\mu\text{g ml}^{-1}$  for *E. coli* and 5  $\mu\text{g ml}^{-1}$  for *L. lactis*.

### 2.3 DNA techniques

Unless otherwise stated, standard procedures were used for DNA manipulation and *E. coli* transformation [26]. Chromosomal DNA from *L. lactis* was isolated as described for *E. coli*, with the modification that the cells were treated with 20  $\text{mg ml}^{-1}$  lysozyme for 2 h. *L. lactis* was made electro-competent as described previously by Holo and Nes [27] with the following modifications: the cells were grown with 1% glycine, and at an optical density of 0.5 (600 nm) ampicillin was added to a final concentration of 20  $\mu\text{g ml}^{-1}$  and incubation was continued at 30°C for 30 minutes. *L. lactis* was transformed as previously described [27]. PCR was performed with DreamTaq DNA polymerase (Fermentas, US) for analytical purposes, or Pfu7x [28] for cloning. Oligonucleotides used are listed in Table S2.

### 2.4 Deletion of *arcA*, *ptk* and *pta*

To delete the *arcA* gene, plasmid pKV11 was used. This plasmid was assembled by using USER™ cloning technology [29]. The upstream and downstream regions of *arcA* were amplified by PCR, using primers p65/p66 and p67/p68, respectively, and the pG<sup>+</sup>host8 DNA fragment was amplified with p63/p64. USER cloning was performed as previously described [30] and pKV11 was obtained by introducing the pKV11 USER mix in chemically competent *E. coli* MC1000. Subsequently pKV11 was introduced in *L. lactis* KF147 and the *arcA* gene was deleted in a double crossover event as described previously [31], resulting in strain KV70. In a similar procedure, the *ptk* gene was deleted using pKV12. The plasmid was constructed by cloning of the upstream and downstream regions of *ptk* obtained in PCR reactions with the primer sets p84/p85 and p86/p87. Transformation of KV70 with

pKV12 resulted in strain KV86. In strain JC119 the *pta* gene was deleted in a similar way using primers p90/p91 and p92/p93.

## 2.5 Introduction of an additional copy of *ptk* in the KF147 $\Delta arcA$ strain

USER™ cloning was used for constructing the plasmid for inserting the *attB* site in KV70. The upstream and downstream regions of *comGC* and the linearized pG<sup>+</sup>host4 vector were obtained by PCR using the primer sets p121/p122, p123/p124, and p64/p109, respectively. The ligated USER mix was introduced into electro-competent *E. coli* MC1000 resulting in plasmid pKV17. Plasmid pKV17 was transformed into KV70 ( $\Delta arcA$ ) and the *attB* site introduced by a double crossover integration event. The resulting strain KV148 was subsequently transformed with pLB65 resulting in strain ASM7, which was used as host for the site-specific integration of the *ptk* gene. The *ptk* gene was inserted with its wild type promoter. A DNA fragment with the *ptk* gene was obtained by PCR with the primer set p130/p131. The PCR product was digested with XbaI/PstI and ligated with pLB85 digested with the same restriction enzymes before introduction into electro-competent *E. coli* ABLE-C. The resulting plasmid pKV20 was inserted on the chromosome of ASM7 ( $\Delta arcA$ , *attB*<sup>+</sup>// pLB65) by site specific-integration resulting in strain ASM39 ( $\Delta arcA$ , *attB*::*ptk*).

## 2.6 Construction of synthetic promoter libraries

Previously we have generated a library of strains based on *L. lactis* MG1363 that express codon-optimized versions of the *Z. mobilis* genes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADHB) from chromosome [32]. Chromosomal DNA from one of these strains was used as template for amplifying a *pdc-adhB* fragment using primers p140 and p141, which was ligated to plasmid pTD6 [32], amplified using primers p150 and p151. The ligation mixture was transformed directly into MG1363. Since the *E. coli gusA* gene encoding  $\beta$ -glucuronidase was incorporated into the downstream of the fusion *pdc-adhB*, we picked up 12 blue colonies after 2 days and named Eth001 to Eth012, respectively. Eth001-Eth012 were cultivated in M17 medium with 45 mM glucose, the GUSA activities in Miller units [32] and ethanol concentrations after 24 h fermentation were used to evaluate the promoter strength. Plasmids from strains efficiently producing ethanol were extracted and introduced into JC119, thus generating the strains JMK001 and JMK002.



## 2.7 Flux determination

Growth was followed by regular measurements of optical density at 600 nm ( $OD_{600}$ ). For analysis of xylose consumption and product formation, high-pressure liquid chromatography (HPLC) was used, where samples of the fermentation broth were extracted and cells were removed by filtration using 0.22  $\mu\text{m}$  filters. The HPLC analysis was performed with an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA) equipped with an Aminex HPX-87H column (BioRad, Hercules, CA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The mobile phase was 5 mM  $\text{H}_2\text{SO}_4$  at a flow rate of 0.6  $\text{ml min}^{-1}$  and column temperature was set to 60°C. In order to be able to calculate the xylose and product fluxes, the cell dry weight (CDW) had to be determined and for this purpose *L. lactis* KF147 and the derivatives KV70 ( $\Delta arcA$ ) and KV86 ( $\Delta arcA, \Delta ptk$ ) were grown exponentially. At an  $OD_{600}$  of 0.4, cells were harvested by filtering through a pre-weighed glass microfiber filter (Whatmann cat. no. 1825-047). Subsequently, the filters were washed with water and dried at 85°C overnight. The CDW was estimated for cells grown with 0.2% xylose to 0.23, 0.26 and 0.27 g CDW per liter of SALM medium per  $OD_{600}$  unit for KF147, KV70 ( $\Delta arcA$ ) and KV86 ( $\Delta arcA, \Delta ptk$ ), respectively. Only small differences in CDW for either of the strains were found when grown with 1.0% and 3.0% xylose (0.28, 0.29 and 0.32 g CDW per liter for KF147, KV70 ( $\Delta arcA$ ) and KV86 ( $\Delta arcA, \Delta ptk$ ), respectively). For calculating the fluxes in ASM39 ( $\Delta arcA, attB::ptk$ ) it was assumed that the CDW was equal to that of KV70 ( $\Delta arcA$ ). For calculating the fluxes in KF147 grown with different concentrations of arginine it was assumed that the CDW was equal to that of KF147 grown in normal SALM with 1.1 mM. Flux was calculated as the product formation per optical density times the specific growth rate divided by the determined CDW.

## 2.8 Calculation of flux through the phosphoketolase pathway and pentose phosphate pathway

Acetate in *L. lactis* is formed from acetyl-P, either generated directly by the phosphoketolase pathway or from acetyl-CoA generated by the pyruvate-formate lyase. Pyruvate-formate lyase generates one formate for every acetyl-CoA, and due to redox constraints only half of the acetyl-CoA is converted into acetate whereas the other half is reduced to ethanol. All acetyl-P generated by the phosphoketolase pathway ends up as acetate, and consequently, the phosphoketolase flux ( $J_{PK}$ ) in xylose equivalents can

be calculated as  $J_{PK} = J_{Acetate} - 0.5 * J_{Formate}$ . The flux through the pentose phosphate pathway ( $J_{PP}$ ) can be calculated as  $J_{PP} = (J_{Lactate} + J_{Formate} - J_{PK}) * 3/5$  as the total glycolytic flux is  $J_{Lactate} + J_{Formate}$  and the contribution from phosphoketolase pathway to this is  $J_{PK}$ , which is subtracted in order to achieve the non-phosphoketolase derived glycolytic flux. Since three xylose molecules end up as 5 GAP when the pentose phosphate pathway is involved (Fig. 1) the multiplication factor 3/5 arises in order to get the fluxes in xylose equivalents.

### 3 Results

#### 3.1 *L. lactis* KF147 co-metabolizes xylose and arginine

It has previously been observed that KF147 can metabolize xylose [20], but never clearly demonstrated whether xylose can be used as the sole energy source. To clarify this, KF147 was inoculated into the chemically defined SALM medium with 0.2%, 1% or 3% xylose and growth was observed in all cases. Irrespective of the xylose concentrations used, a distinct two-phased growth pattern was observed, where the specific growth in the second phase was slower (Fig. 2, Table 1). There was no apparent difference in the growth profile between the cultures with 1% and 3% xylose, but low xylose concentration (0.2 %) resulted in significantly reduced growth rates in both phases (Fig. 2, Table 1). *L. lactis* efficiently metabolize arginine when growing on non-PTS sugars [35]. Therefore, we speculated that the presence of the two phases could be attributed to arginine being catabolized, since the SALM medium contains arginine that could be utilized through the arginine deiminase pathway generating ATP (Fig. 2). An amino acid analysis of the medium before and after the shift in growth rate confirmed that arginine indeed was metabolized and that the shift correlated with the depletion of arginine. Furthermore we found that the concentration of arginine in the medium had an influence on the specific growth rate and fermentation product profile. At low xylose concentration (0.2%), the specific growth rate increased with the concentration of arginine, while this correlation was absent at high xylose concentration (Table S3). The xylose flux decreased with increasing arginine concentrations for all xylose concentrations, but no significant differences in the yields of lactate, formate, acetate and ethanol were observed, irrespective of xylose concentration (Table S3).

To simplify further studies, arginine catabolism was effectively eliminated by deleting the gene encoding arginine deiminase (*arcA*) resulting in strain KV70. As seen in Fig. 2C by deleting *arcA* the two-phased growth was eliminated and arginine was no longer depleted.

### 3.2 Two different pathways for metabolizing xylose are operating in *L. lactis* KF147

To determine how xylose is metabolized in *L. lactis* KF147, the strain KV70 ( $\Delta arcA$ ) was grown in the defined SALM medium supplied with 0.2%, 1% and 3% (w/v) xylose. At high xylose concentrations (1% or 3%) the final cell density, as expected, was higher than at a low concentration (0.2%) (Fig. 2B), and growth ceased due to acidification of the medium, and not because of depletion of xylose (data not shown). With 0.2% xylose the pH of the medium was not affected, and growth ceased due to xylose depletion (data not shown). The specific growth rates and the xylose and product fluxes were determined as shown in Table 1 and 2. The specific growth rate for KV70 ( $\Delta arcA$ ) was reduced at 0.2% xylose and the end-products observed were lactate, formate, acetate and ethanol. Increased fluxes were observed with increasing xylose concentrations (Table 2). For a sugar that is solely metabolized through glycolysis, the formate:acetate:ethanol ratio should be close to 2:1:1, since each pyruvate formed results in one NADH, which in turn allows for the reduction of half the acetyl-CoA formed by the pyruvate formate lyase (PFL) (Fig. 1). The ratio observed here was approximately 2:2:1 at all xylose concentrations, which indicates that the phosphoketolase pathway is active in KF147. The fluxes through the phosphoketolase pathway and pentose phosphate pathway were subsequently calculated, and the proportion of xylose metabolized through the phosphoketolase pathway ( $Q_{PK}$ ) was determined (Table 2). The ratio between lactate and mixed-acids ( $J_{Lac}/J_{For}$ ) was determined as the ratio between the flux of lactate and formate. With increasing xylose concentrations we found that the flux through the phosphoketolase pathway remained almost constant, whereas the flux through the pentose phosphate pathway increased. The carbon recovery at 0.2% and 1% xylose were both above 90%, whereas it was around 83% at 3% xylose. The  $Q_{PK}$  decreased with the increase in xylose concentrations, whereas  $J_{Lac}/J_{For}$  increased. This finding clearly demonstrate that xylose is metabolized through both the phosphoketolase pathway and the pentose phosphate pathway in KF147, and that a

shift occurs from the phosphoketolase pathway towards the pentose phosphate pathway as the concentration of xylose increases.

### 3.3 Inactivation of the phosphoketolase pathway and its effects on growth and product formation

To further corroborate the above conclusion that xylose is metabolized through both pathways, the phosphoketolase pathway was inactivated by deleting *ptk* in KV70 ( $\Delta arcA$ ), thus giving rise to KV86 ( $\Delta arcA, \Delta ptk$ ). KV86 was then characterized in the same way as above in SALM medium supplemented with either 0.2%, 1% or 3% xylose. No significant difference in the specific growth rates of KV86 and KV70 was found at the low xylose concentration ( $0.23 \pm 0.01 \text{ hr}^{-1}$  and  $0.25 \pm 0.01 \text{ hr}^{-1}$ ) but KV86 grew slower at the high xylose concentrations when compared to KV70 (Table 1). As previously observed for KV70, the specific growth rate for KV86 at 1% and 3% xylose was identical (Table 1). The same products as before were observed (Table 2), but the formate:acetate:ethanol ratio was now very close to 2:1:1 as expected from the redox constraints. As for the parent strain the carbon recovery was above 90% at 0.2% and 1% xylose and 86% at 3% xylose. Inactivating the phosphoketolase pathway resulted in an increase in the lactate flux at the higher xylose concentrations (1% and 3%) and, at all xylose concentrations a higher  $J_{\text{Lac}}/J_{\text{For}}$  ratio was observed (Table 2). Thus deletion of the phosphoketolase pathway resulted in a more homolactic fermentation pattern, but the main fermentation products were still formate, acetate and ethanol.

### 3.4 Increased phosphoketolase activity leads to an increase in the flux through the phosphoketolase pathway

Inactivation of the phosphoketolase pathway by deleting the *ptk* gene resulted in a more homolactic fermentation profile. To study the effect of overexpressing the phosphoketolase pathway, an additional copy of *ptk* transcribed from its own promoter was introduced in the chromosome by site-specific integration into an *attB* site using the TP901-1 bacteriophage integrase [21]. Inspection of *L. lactis* KF147 genome sequence revealed that it does not contain an appropriate *attB* site [15]. Therefore, the *attB* site from MG1363 was first introduced by homologous recombination, generating KV148 ( $\Delta arcA, attB^+$ ). Subsequently plasmid pLB65, carrying the *int* gene encoding the TP901-1

integrase, was introduced, resulting in strain ASM7 ( $\Delta arcA$ ,  $attB^+$ //pLB65) into which the *ptk* gene was inserted into the *attB* site thus creating ASM39 ( $\Delta arcA$ ,  $attB::ptk$ ). ASM39 was characterized in the same way as above with respect to specific growth rate and fluxes in SALM medium supplemented with 3% xylose. A slight reduction in the growth rate of ASM39, when compared to the parent strain, was observed ( $0.33 \pm 0.00 \text{ h}^{-1}$  versus  $0.36 \pm 0.01 \text{ h}^{-1}$ ). As for the parent strain the fermentation products were lactate, formate, acetate and ethanol, however, overall the product fluxes were smaller (Table 2). The formate:acetate:ethanol ratio was approximately 2:2:1 and the carbon recovery for ASM39 (KF147  $\Delta arcA$ ,  $attB::ptk$ ) was around 90%. The effect of inserting an additional copy of *ptk* was an increased flux through the phosphoketolase pathway (from  $7.7 \pm 1.4$  to  $11.9 \pm 0.2$ ) and the fraction of xylose metabolized through the phosphoketolase pathway,  $Q_{PK}$ , was increased 1.8 fold (Table 2). Correspondingly, the  $J_{Lac}/J_{For}$  ratio was significantly lower, which is in accordance with a reduced lactate flux ( $0.13 \pm 0.1$  versus  $0.05 \pm 0.0$ ). In conclusion, overexpression of the phosphoketolase pathway resulted in a shift towards a more mixed-acid fermentation pattern.

### 3.5 Redirecting metabolism towards ethanol production

After characterizing xylose metabolism in KF147 we attempted to redirect metabolism towards ethanol, to demonstrate its potential as a platform for converting xylose containing feedstocks. First we determined the ethanol tolerance of KF147 (Fig. 3A) and we found that the specific growth rate was reduced from  $0.70 \text{ h}^{-1}$  without ethanol to  $0.48 \text{ h}^{-1}$  in the presence of 4% ethanol (vol/vol) and  $0.12 \text{ h}^{-1}$  under 6% ethanol (vol/vol). Then we inactivated *pta*, encoding the phosphotransacetylase, in KV86 to obtain the strain JC119 ( $\Delta arcA \Delta ptk \Delta pta$ ), where the acetate flux had almost completely been blocked (Table 3).

The next step involved introducing pyruvate decarboxylase and alcohol dehydrogenase, where we decided to use synthetic codon-optimized versions of the *Z. mobilis* genes. First a library of strains was generated where the genes were expressed from a plasmid using different synthetic promoters and transcriptionally fused to the *gusA* reporter gene encoding  $\beta$ -glucuronidase. 12 candidate strains were selected, Eth001-Eth012, that appeared to have the ethanol genes expressed to different levels. Subsequently, we measured their GUSA activities and determined the product formation for these

strains grown in rich medium containing glucose. It was found that Eth001 and Eth006, which have higher GUSA activities, were the most efficient ethanol producers (Fig 3B). Plasmids from these two strains were extracted (pEth001 and pEth006) and the sequence of their synthetic promoters can be seen in Fig. 3C.

Subsequently, pEth001 and pEth006 were transformed to JC119 ( $\Delta arcA \Delta ptk \Delta pta$ ) to obtain JMK002 and JMK001, and the strains were characterized in xylose containing defined medium. For JMK002, the concentrations of lactate, formate, acetate and acetoin had been significantly reduced when compared to JC119 ( $\Delta arcA \Delta ptk \Delta pta$ ), and for the strain with highest expression of the ethanol genes, JMK001, almost all the xylose ended up as ethanol, and the ethanol yield reached 83% of the theoretical maximum (Table 3).

#### 4 Discussion

Previously, Tanaka et al. reported that when the non-dairy *L. lactis* IO-1 metabolizes xylose through the pentose phosphate pathway, only lactate is formed, whereas metabolism through the phosphoketolase pathway results in formation of acetate, formate and ethanol [14]. For *L. lactis* KF147, we found that only a small fraction of the xylose that enters the pentose phosphate pathway ends up as lactate, and that much less lactate is formed than for *L. lactis* IO-1. Our first assumption was that this probably was due to the use of a synthetic defined medium in this study, where Tanaka et al. used the rich M17 medium. Also we used a lower incubation temperature, which has been shown to influence product formation. Therefore, we carefully compared IO-1 and KF147 in M17 at different temperatures. The results of this comparison can be seen in the supplementary materials. From Table S4 it is apparent that there is an increase in lactate yield when the strains are grown in rich M17 medium. At 30°C KF147 and IO-1 produced equal amounts of lactate, however, at 37°C, surprisingly, KF147 was more homolactic than IO-1. This switch towards a more homolactic fermentation profile in M17 probably is due to a reduced ATP demand when growing on a rich substrate, where most of the building blocks are available, and therefore less ATP is needed for anabolism. This has also been observed previously for re-suspended cells of *L. lactis* [33]. IO-1 has been demonstrated to show

potential as a cell factory for production of lactate and other value-added products, however, obvious drawbacks of this strain are that it is difficult to engineer [34] and that it cannot utilize arabinose. In this study we have successfully carried out several genetic modifications of another non-dairy isolate - KF147, including deleting and integrating genes by using genetic tools not previously applied on KF147.

When studying xylose metabolism in KF147 we used a defined medium, which allowed us to achieve balanced growth, and made it possible to determine substrate and product fluxes with great accuracy, which is not always possible when using complex media. We found that arginine was co-metabolized when KF147 was grown on xylose, which stimulated growth and cell yield, and this phenomenon has also been shown take place when *L. lactis* is grown on other slowly metabolized carbohydrates [35]. Arginine is catabolized through the arginine deiminase pathway and results in ATP formation. The arginine deiminase pathway has previously been predicted to be present in *L. lactis* KF147 [15], and with this work we have demonstrated that it indeed is functional. The interference from arginine could easily be removed by a simple deletion of the *arcA* gene encoding the arginine deiminase.

The flux analysis of KF147 suggested that during exponential growth, xylose was metabolized through both the pentose phosphate pathway and the phosphoketolase pathway. This was confirmed by both deleting and overexpressing the phosphoketolase. When the phosphoketolase was deleted an increase in the lactate production was observed; however the main products were still formate, acetate and ethanol. In contrast, a significant decrease in lactate production was observed when the phosphoketolase was overexpressed. We found that at increased xylose concentrations the metabolism shifted towards the pentose phosphate pathway, and that the shift was more prominent when arginine could be catabolized (KF147  $\Delta arcA$  versus KF147 first growth phase). Without arginine available as an ATP source, the cells apparently could compensate by increasing the rate at which xylose is catabolized, and this was observed at all xylose concentrations (KF147  $\Delta arcA$  versus KF147 first growth phase). We also found that KF147 grew slower after depleting arginine than the non-catabolizing KF147  $\Delta arcA$  strain. The slow growth observed for KF147 in the second phase could be



the result of arginine starvation as externally supplied arginine is no longer available for synthesis of proteins.

When the phosphoketolase pathway is used, 2 moles of ATP (one formed from acetyl-phosphate, and two in glycolysis minus the one consumed when X-5-P is formed) and 1 mole of pyruvate are formed from one mole of xylose, whereas only 1.67 moles of ATP ( $9/3$  in glycolysis,  $-1/3$  in modified pentose phosphate pathway, and one consumed when X-5-P is formed) and 1.67 moles of pyruvate are formed when the pentose pathway is taken. The fate of pyruvate decides the overall ATP yield, and for the two pathways an identical yield is obtained in the absence of lactate formation. It is interesting to compare growth and product formation of KV70 ( $\Delta arcA$ ) and KV86 ( $\Delta arcA \Delta ptk$ ). At all xylose concentrations KV70 grew faster than KV86 and KV86 formed more lactate than KV70, which results in a poorer ATP supply for KV86 than for KV70. This could be explained by the presence of a bottleneck in the mixed-acid pathway as the formate flux is more or less similar for the two strains. A higher formate flux would result in more ATP being synthesized by the acetate kinase, which potentially could speed up growth. A similar phenomenon was observed when the phosphoketolase pathway was inactivated in an *L. lactis* IL1403 derivative genetically modified to grow on xylose [36] and here the researchers found that it was possible to restore growth by over-expressing the transketolase. IL1403 is however quite different from KF147 in that it has a much less active mixed-acid pathway and remains quite homolactic, even on slowly metabolizable sugars such as maltose and galactose [37]. It is quite possible that the factors limiting growth of KV86 on xylose could be different from those of a genetically engineered dairy isolate that naturally is unable to ferment xylose.

As a demonstration of the potential of KF147 to serve as a cell factory in second generation biorefineries, we successfully rewired its metabolism into homo-ethanol production. We have previously engineered the dairy isolate into producing ethanol as the sole fermentation product from a range of sugars [7, 32], and based on this knowledge we successfully managed to change the fermentation pattern of KF147 on xylose to one where ethanol was the sole product. Surprisingly this could be achieved without inactivating the lactate dehydrogenase, which probably is necessary to do when the feedstock is comprised of other sugars in addition to xylose. This is beyond the scope of the



current study which focuses on xylose but it is manageable, since we have demonstrated that KF147 is easy to manipulate genetically, and we have achieved this for other *L. lactis* strains in the past [38, 39, 42]. The advantages of using *L. lactis* KF147 as a cell factory include its high growth rate on xylose (around 0.35 h<sup>-1</sup>), which is significantly higher than the growth rate (around 0.10 h<sup>-1</sup>) of the engineered *Saccharomyces cerevisiae* [40, 41], and the native metabolism of other pentoses (such as arabinose). Further the ethanol tolerance can be easily improved [32]. While one potential challenge which we currently are dealing with is catabolite repression. It would be desirable if glucose and xylose, two of the common sugars found in lignocellulose hydrolysates, could be co-metabolized, and this can probably be accomplished simply by replacing the promoters expressing the xylose related genes with ones that are non-regulated. Another challenge lies in the selection of economically feasible feedstocks (including carbon and nitrogen sources) for the development of efficient processes.

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#### **Conflict of interest**

The authors declare no competing financial interests.

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**Table 1.** The specific growth rates of *L. lactis* KF147 and its derivatives

	$\mu$ (h <sup>-1</sup> )		
	0.2% xylose	1% xylose	3% xylose
KF147 first growth phase <sup>a)</sup>	0.28 ±0.01	0.40 ±0.01	0.39 ±0.02
KF147 second growth phase <sup>b)</sup>	0.15 ±0.01	0.26 ±0.01	0.28 ±0.01
KV70 ( $\Delta arcA$ )	0.25 ±0.01	0.36 ±0.02	0.36 ±0.01
KV86 ( $\Delta arcA, \Delta ptk$ )	0.23 ±0.01	0.31 ±0.02	0.31 ±0.01

a) 0 to 7 hours growth (Figure 2A) - exogenous arginine present in the medium

b) 7 to 12 hours growth (Figure 2A) - exogenous arginine depleted. Values are averages of data derived from at least three independent experiments.

**Table 2.** The xylose and product fluxes for *L. lactis* KF147 and its derivatives

Xylose conc.	Flux (mmol/h-gDW)					$J_{Lac}/J_{For}$ <sup>a)</sup>	$J_{PK}$	$J_{PP}$	$Q_{PK}$ <sup>b)</sup>
	Xylose	Lactate	Formate	Acetate	Ethanol				
<b>KV70 - KF147 <math>\Delta arcA</math></b>									
0.2%	16.4±0.8	0.5±0.1	19.1±0.8	18.4±1.1	9.2±0.8	0.02±0.00	8.8±0.5	6.5±0.5	0.58±0.03
1%	19.8±1.5	2.4±0.4	22.5±1.2	19.1±1.3	9.8±1.2	0.11±0.02	7.8±1.4	10.3±1.3	0.43±0.07
3%	23.6±3.4	3.1±1.7	23.9±1.2	19.6±1.4	11.7±0.8	0.13±0.08	7.7±1.4	11.6±1.1	0.40±0.06
<b>KV86 - KF147 <math>\Delta arcA \Delta ptk</math></b>									
0.2%	15.8±1.2	0.6±0.1	23.8±0.5	13.0±0.1	12.0±0.4	0.03±0.00	-	13.9±0.4	-
1%	17.9±1.0	3.7±0.3	20.9±1.5	10.8±0.8	9.9±1.5	0.18±0.02	-	14.6±1.0	-
3%	21.6±1.9	8.7±1.1	21.9±0.2	10.9±0.2	11.3±0.2	0.40±0.08	-	18.3±0.8	-
<b>ASM39 - KF147 <math>\Delta arcA, attB::ptk</math></b>									
3%	18.5±0.5	0.9±0.1	18.9±0.1	21.4±0.2	10.6±0.6	0.05±0.00	11.9±0.2	4.7±0.2	0.72±0.01
<b>KF147 - First growth phase with arginine</b>									
0.2%	13.2±0.8	0.8±0.1	16.7±0.7	16.1±0.7	7.2±0.8	0.05±0.00	7.7±0.8	5.8±0.8	0.57±0.06
1%	14.5±1.1	2.1±0.3	19.0±1.2	14.4±0.8	8.6±1.7	0.11±0.02	4.9±0.3	9.7±0.4	0.34±0.01
3%	19.4±1.8	7.3±2.0	20.8±2.4	14.8±0.6	10.3±2.0	0.36±0.13	4.5±0.8	14.1±1.0	0.24±0.04
<b>KF147 - Second growth phase without arginine</b>									
0.2%	12.7±0.4	1.1±0.1	15.1±0.7	15.2±0.4	6.7±0.4	0.07±0.01	7.6±0.1	5.2±0.4	0.60±0.02
1%	17.8±1.4	3.5±1.0	18.4±0.5	16.1±0.3	9.7±0.2	0.19±0.05	6.9±0.2	9.0±0.9	0.44±0.03
3%	21.2±1.1	5.3±0.4	19.0±1.6	16.5±1.3	9.1±0.9	0.28±0.04	7.0±0.6	10.4±0.4	0.40±0.01

<sup>a)</sup>  $J_{Lac}/J_{For}$  is the ratio between lactate and mix-acid fermentation.  $J_{PK}$  and  $J_{PP}$  are the calculated flux through respectively the phosphoketolase pathway and pentose phosphate pathway and  $Q_{PK}$  the fraction of xylose metabolized through the phosphoketolase pathway.

<sup>b)</sup>  $Q_{PK} = J_{PK}/(J_{PK} + J_{PP})$ . Values are average of at least three independent experiments.

**Table 3.** Redirection of xylose metabolism towards ethanol production<sup>a)</sup>

Strains	Products (mM)					Carbon Recovery <sup>b)</sup>	Ethanol yield <sup>c)</sup> %
	Lactate	Formate	Acetate	Acetoin	Ethanol		
KV86	18.4 ± 0.3	50.1 ± 0.8	27.9 ± 0.5	ND	18.0 ± 0.2	81	21
JC119	17.9 ± 0.7	22.8 ± 1.4	0.9 ± 0.1	10.9 ± 0.5	19.7 ± 0.6	75	23
JMK002	2.7 ± 0.2	3.8 ± 0.5	1.4 ± 0.3	1.9 ± 0.7	56.3 ± 1.0	75	66
JMK001	ND	ND	ND	ND	70.6 ± 1.5	83	83

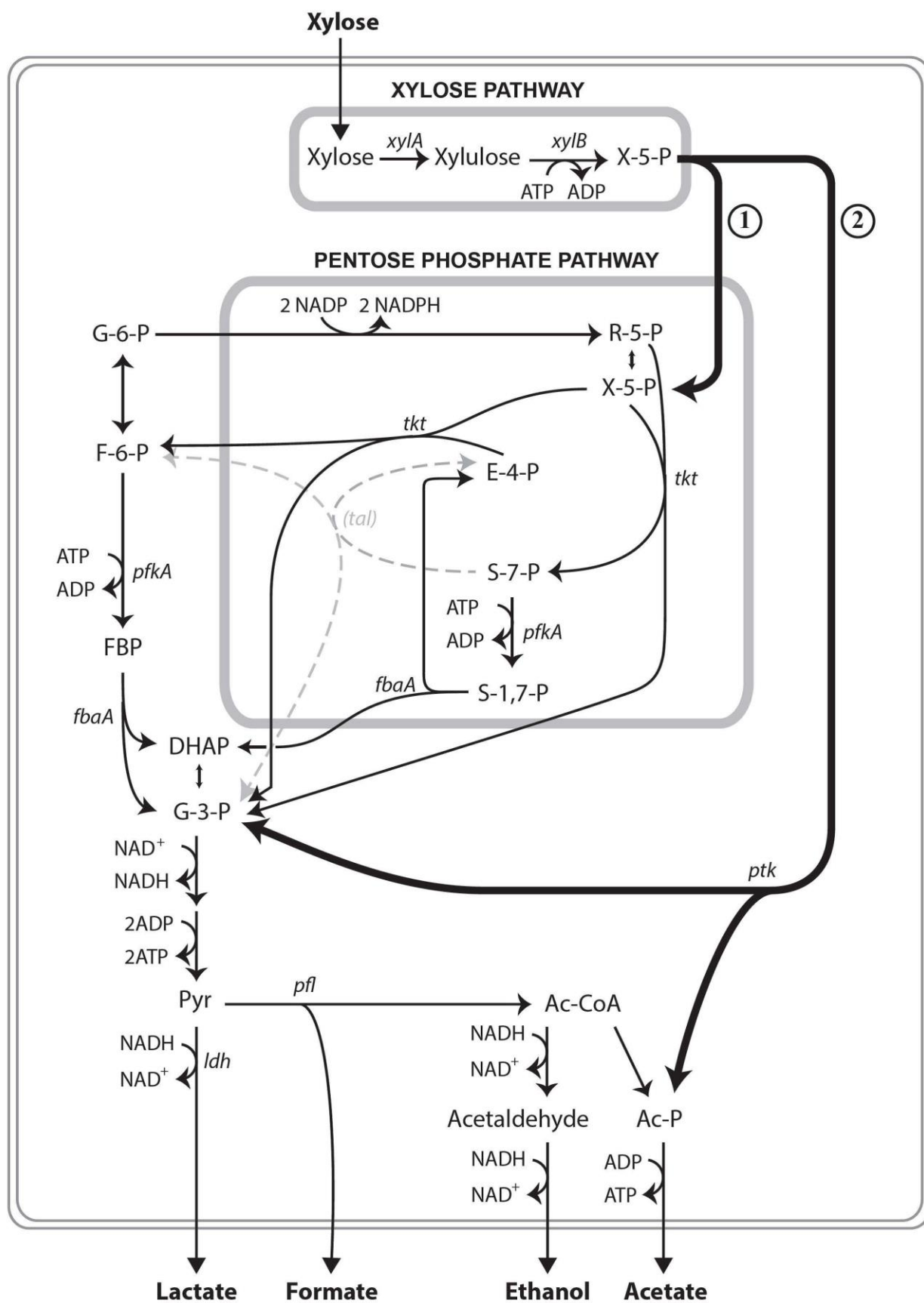
<sup>a)</sup>All the strains were cultivated in SALM medium with 51 mM xylose. The samples were collected after 2 days. Values are averages of data obtained from two independent experiments.

<sup>b)</sup>Carbon recovery was calculated based on the carbon balance and in strain JC119 and JMK002, a small peak of *meso*-(2,3)-butanediol was detected, but not included in the calculation.

<sup>c)</sup>Ethanol yield was calculated based on the maximum theoretical yield of ethanol through PDC/ADHB pathway, which is 1.67 mol ethanol/1 mol xylose.

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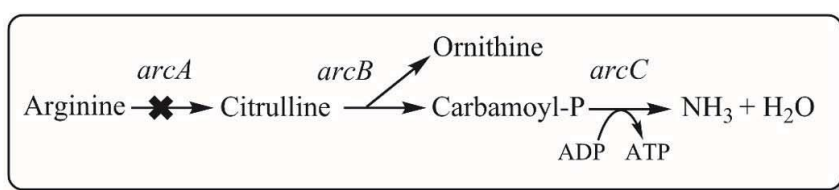
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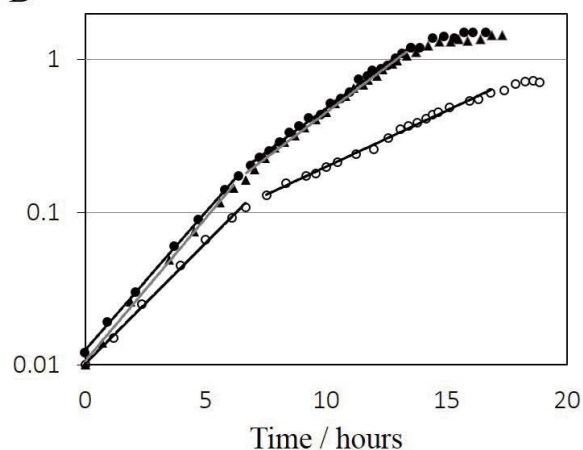


**Figure 1.** Schematic presentation of the xylose catabolism in *L. lactis* KF147. After conversion of xylose to xylulose-5-phosphate (X-5-P) in the xylose pathway, X-5-P can either enter the pentose phosphate pathway ① or the phosphoketolase pathway ②. As no transaldolase is present in *L. lactis*, it has been speculated that phosphofructokinase and aldolase are involved instead in the pentose phosphate pathway. X-5-P: xylulose-5-phosphate, R-5-P: ribose-5-phosphate, G-6-P: glucose-6-phosphate, F-6-P: Fructose-6-phosphate, FBP: fructose 1,6-bisphosphatase, E-4-P: erythrose-4-phosphate, S-7-P: sedoheptulose-7-phosphate, S-1,7-P: sedoheptulose-1,7-bisphosphate, DHAP: Dihydroxyacetone phosphate, G-3-P: glyceraldehyde-3-phosphate, Pyr: pyruvate, Ac-CoA: acetyl-CoA and Ac-P: acetyl-phosphate. *xylA*: xylose isomerase, *xylB*: xylulokinase, *tkt*: transketolase, *tal*: transaldolase, *pfkA*: 6-phosphofructokinase, *fbaA*: fructose-bisphosphate, *ptk*: phosphoketolase, *pfl*: pyruvate-formate lyase, *ldh*: L-lactate dehydrogenase

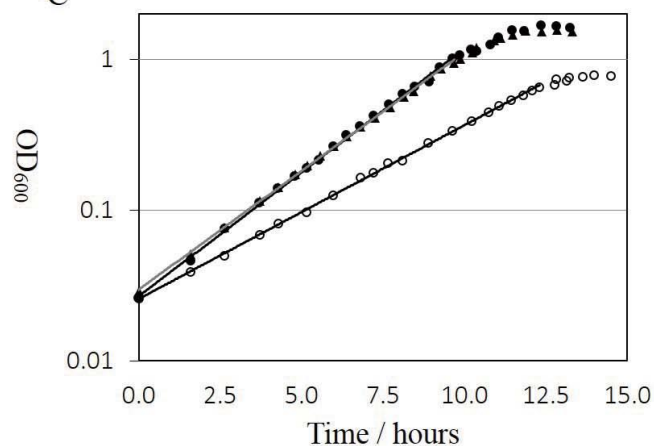
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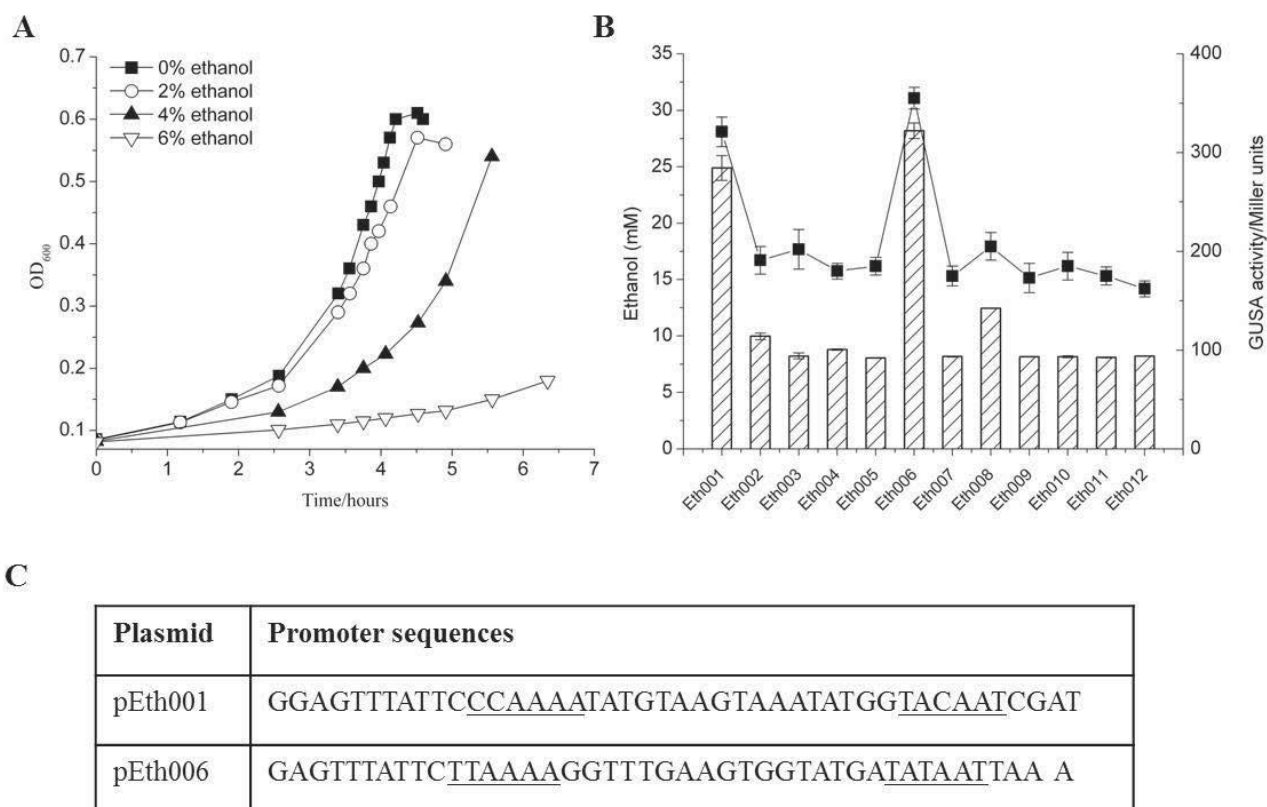
B



C



**Figure 2.** An overview of arginine catabolism (A) & growth KF147 (B) and its  $\Delta arcA$  (C) derivative in defined SALM medium. The strains were grown in defined SALM medium supplemented with 0.2% (open circle), 1% (filled circle) or 3% xylose (filled triangle). One representative growth curve from three independent experiments for each stain is shown.



**Figure 3.** Construction of synthetic promoter libraries for driving the expression of PDC/ADHB. **(A)** The ethanol tolerance of KF147 in defined SALM medium with 0.2% glucose. The ethanol concentration was in vol/vol. **(B)** GUSA activity and ethanol concentration were measured for each strain. The method for determining GUSA activity was described in Ref. [32]. The strains Eth001-Eth012 were grown in M17 medium with 45 mM glucose and cultivation medium was analyzed after 24 h for to determine ethanol concentration. The error bars indicate the standard deviation which is based on two replicates **(C)** The sequences of the promoters driving expression of the ethanol genes in plasmid pEth001 and pEth006.