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METABOLIC CAPABILITIES OF *Actinobacillus succinogenes* FOR SUCCINIC ACID PRODUCTION

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Abstract - Attention has been focused on microbial succinic acid production as an alternative for conventional chemical synthesis that is associated with environmental pollution. A metabolic model for *Actinobacillus succinogenes* 130Z was developed with a mixture of glucose and xylose as substrate. The metabolic fluxes during succinicate production were determined using flux balance analysis by linear programming optimization in the MATLAB environment. Different glucose ratios (0.3, 0.4 and 0.7 mol.mol^{-1}substrate) were used as model assumptions to calculate optimal fluxes, maximum growth and succinate production. The model revealed that higher growth rates and product yields were correlated with higher glucose content in the substrate mixture. When glucose constituted 0.5 mol.mol^{-1} substrate, a lower succinate yield (0.64 mol.mol^{-1} substrate) was obtained, compared to 0.73 mol.mol^{-1} substrate when glucose was used individually. Deletion of different unessential reactions in the model showed that a knockout of the acetate formation pathway would increase the succinate yield by 21% when glucose and xylose were used in equal molar ratios.

Keywords: Succinic Acid; *Actinobacillus succinogenes*; Xylose; Metabolic Model.

INTRODUCTION

Succinic acid is a valuable product which is used in food, pharmaceutical, agricultural and, above all, petrochemical industries (McKinlay *et al*., 2007a). It is an important building block with a great economical potential and a precursor for other valuable chemicals. *Actinobacillus succinogenes*, a facultative anaerobic member of the *Pasteurellaceae* family, is one of the most promising microorganisms for industrial applications due to its high productivity, and the ability of growth on a broad range of substrates (Beauprez *et al*., 2010). Recent studies have been conducted using renewable cheap carbon sources for succinic acid production by *A .succinogenes* to reduce the cost of the end product. Moreover, many of these sources are waste made by other industries and reusing them will lead to reducing environmental pollution. Different sources has been used as renewable carbon substrate, including corn stalk and cotton stalk (Li *et al*., 2010), cane molasses (Liu *et al*., 2008), sugarcane baggase (Borges and Pereira, 2011), straw hydrolysate (Zheng *et al*., 2009), corn stover (Zheng *et al*., 2010), sake lees (Chen *et al*., 2010), corn fiber (Li *et al*., 2011) and waste yeast hydrolysate (Chen *et al*., 2011). Many of these cheap renewable sources are mixtures of hexose and pentose after depolymerisation (Jeffries and Jin, 2004). Therefore, metabolic studies should be carried out in order to show the maximum metabolic capabilities of *A. succinogenes* when it is grown on a mixture of xylose and glucose obtained from renewable sources.

Rational metabolic engineering strategies can be offered only when a comprehensive understanding of

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metabolic pathways has been provided (McKinlay *et al*., 2007). Knowledge resulting from flux analysis studies could be utilized to conduct metabolic engineering strategies for designing high producer strains, which are of great importance in industrial processes.

The nature of the carbon substrate is a significant factor that affects directly the carbon flux distribution, succinic acid yields and byproduct formation. Using metabolic engineering to produce *A. succinogenes* strains in which the carbon flux is directed to succinate production pathway instead of acetate and formate is desirable for industrial production of succinic acid due to higher productivity and subsequently lower end product yields.

In this study, the effect of glucose and xylose (individually and in mixtures) as carbon substrate on flux distribution and succinate yield in *A. succinogenes* has been investigated. In addition, the effect of different knockouts in the metabolic network on product yields and the growth rate has been studied.

MATERIALS AND METHODS

Metabolic Model for *A. succinogenes* 130Z

In order to perform metabolic engineering studies, a system of metabolic reactions should be developed that can be solved with linear programming optimization to give optimum fluxes and information about products. Therefore, a set of reactions was collected based on the stoichiometry and enzymes that have been determined to be active *in vitro*, as shown in Table 1(McKinlay *et al*., 2007; McKinlay *et al*., 2010). In flux balance analysis (FBA) studies, all the intracellular metabolites and cofactors should be taken into account in the model. The information about reversibility or irreversibility of reactions has been extracted from the KEGG database (http://www.kegg.com) and was used as additional constraints. The metabolic model was developed using 21 intracellular metabolites, 7 extracellular metabolites and 27 reactions. A list of reactions used in the *A. succinogenes* metabolic model is given in Table 1.

Table 1: A list of metabolic reactions included in the metabolic model of *A. succinogenes* (McKinlay *et al*., 2007; McKinlay *et al*., 2010).

Glycolysis	**Pentose Phosphate**
GLC+ATP → G6P	G6P↔2NADPH+RU5P +CO2
F6P↔G6P	RU5P↔R5P
F6P+ATP→F1,6P	R5P+X5P↔GA3P+S7P
F1,6P↔DHAP+GA3P	GA3P+S7P→E4P+F6P
DHAP↔GA3P	E4P+X5P→F6P+GA3P
GA3P↔1,3PG+NADH +ATP	
1,3PG↔3PG + ATP	**TCA Cycle**
3PG↔2PG	
2PG↔PEP	PEP+CO2↔OAA+ATP
PEP↔PYR +ATP	OAA→PYR+CO2
	OAA+NADH↔MAL
Xylose Catabolism	MAL→PYR+NADPH+CO2
	MAL↔FUM
Xyl+ATP→X5P	FUM+NADH+2/3 ATP↔SUC
X5P↔RU5P	
	Biomass Formation
Byproduct Formation	
	13.49NADPH+0.00041G6P+0.000126F6P+0.000686R5P+0.000099
PYR→AcCoA+ NADH +FOR	G3P+0.0013703PG+0.000528pep+0.002764Pyr+0.003006AcCoA
AcCoA→ACE +ATP	+0.001502 OAA+0.046930ATP → Bio +0.002727NADH
AcCoA+2NADH→ETH	
Transhydrogenation	
NADPH↔NADH	

Flux Balance Analysis (FBA)

FBA utilizes a linear programming optimization method to determine what the maximum capabilities of an organism are in steady state conditions for a given carbon substrate. It analyzes the optimum flux distribution in order to maximize or minimize an objective function (Orth *et al*., 2010). In addition, FBA models are profitable for analyzing the effect of environmental conditions on flux distribution and metabolite synthesis. The first step is to collect the metabolic reactions of the microorganism. In steady state conditions the total amount of each intracellular metabolite being produced is equal to the amount of that metabolite being consumed. Therefore, writing a mass balance for each intracellular metabolite leads to Equation (1), in which S and V are the stoichiometry coefficient matrix for metabolic reactions and reaction fluxes in steady state conditions, respectively. This equation is the basis of FBA models.

$$S.V=0$$
$$\text{Max } Z=C_iV_i \quad (1)$$
$$A\leq V\leq B$$

Z is the objective function and A and B are lower and upper constraints for metabolic fluxes. Other essential data which should be determined for use in a FBA model are the growth requirements for the microorganism. For writing the biomass formation equation that shows the required intracellular metabolites for growth, the metabolite requirements were assumed to be the same as those in a previous study (McKinlay *et al*., 2007b). Since the number of reactions usually exceeds the number of metabolites, several constraints should be imposed to give a definite solution. These include upper and lower constraints for each flux (which are considered to be $-a\leq V_i\leq a$ and $0\leq V_i\leq a$ for reversible and irreversible reactions, respectively) and substrate uptake.

Reaction fluxes were calculated by the model using maximization of the growth rate as the objective function and choosing the Linear Programming method in the Optimization toolbox in MATLAB environment.

Different glucose ratios (0.3, 0.5 and 0.7 mol.mol^{-1} substrate), were used for investigation of the flux distribution and succinate formation. Product yields with xylose-glucose mixtures were compared to conditions when xylose and glucose were individual substrates.

Figure 1 shows the central metabolic network of *A. succinogenes* with different glucose ratios for wild type *A. succinogenes*. An incomplete TCA cycle has been recognized for *A. succinogenes* as there is no report of alpha keto glutarate (AKG) production *in vitro* (McKinley *et al*., 2005).

Knockouts and Proposed Mutants

In order to study metabolic engineering strategies for *A. succinogenes* and their effect on succinate yields, different reactions were subjected to deletion, and the effect of inactivation of the corresponding enzymes on growth and product yields was investigated by the metabolic model. Due to the contribution of most intracellular metabolites in the biomass formation equation, there were a few possible mutants which could be proposed and studied by the model.

RESULTS AND DISCUSSION

Effect of Different Glucose Ratios on the Flux Distribution

It has been shown that the simultaneous use of glucose and xylose as substrate not only improves the succinate yield, but also increases xylose utilization (Liu *et al*., 2013). A metabolic model was developed to study the effect of glucose/xylose mixtures as carbon substrate on succinic acid production by *A. succinogenes*.

Figure 1 shows the metabolic fluxes of wild type *A. succinogenes* for three different glucose ratios (0.3, 0.5 and 0.7 mol.mol^{-1} substrate). Succinate was the major product at all glucose/xylose ratios, though formate and acetate were also produced. It can be seen that an increase in the glucose/xylose ratio leads to an increase in both succinate and other byproduct formation. A succinate yield of 0.64 mol.mol^{-1} substrate was predicted by the model when the glucose and xylose ratios were 0.5 mol.mol^{-1} substrate. Increasing the glucose ratio to 0.7 mol.mol^{-1} substrate as a model assumption showed higher product yields in wild type *A. succinogenes*. This is in accordance with an experimental study in which corn straw hydrolysate (containing glucose and xylose) was used as substrate and higher glucose contents in the substrate resulted in higher succinic acid production (Zheng *et al*., 2009). Maximum succinate yields of 0.73 and 0.58 mol.mol^{-1} substrate were predicted by the model when glucose and xylose were the individual substrates, respectively. The succinate yield of 0.73 for glucose was in good agreement with 0.7 mol.mol^{-1} glucose which was reported in a previous study (McKinlay *et al*., 2005).

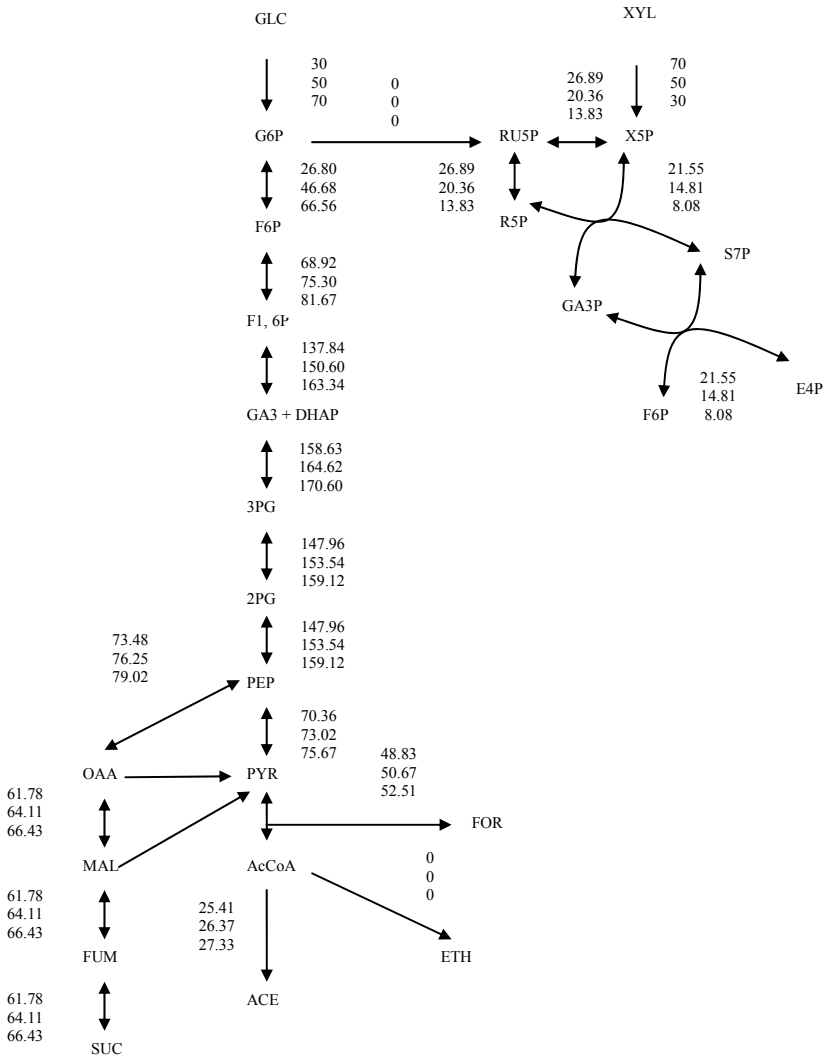

Figure 1: Metabolic fluxes of *A. succinogenes* with different glucose ratios (0.3, 0.5 and 0.7 mol.mol^{-1} substrate, respectively). All the fluxes are calculated per 100 mol substrate.

Enzyme Activity

Increasing the flux through a metabolic pathway means an increase in the activity of the enzymes involved in the pathway reactions (Fell, 2005). Enzyme activities were studied by analyzing the magnitude of the metabolic fluxes at different glucose ratios. Malic enzyme (ME) and malic dehydrogenase (MDH) are two enzymes responsible for switching the carbon flux between succinate and other products (Beauprez *et al*., 2010). The metabolic model recognized no oxaloacetate decarboxylase (OADC), ME and alcohol dehydrogenase (AD) activity in the wild type strain when the maximization of growth rate was the objective function. There were increases in the activity of Pyruvate formate lyase (PFL), acetate kinase (AK) and fumarate reductase (FR) with increasing glucose ratio in wild type *A. succinogenes*. These enzymes contribute to formate, acetate and succinate formation reactions, respectively. Of the three mutants examined by the model, mutant1 had the lowest activity of PFL, which means the minimum formate production compared to other mutants. Interestingly, FR showed higher activity for mutant 1, which subsequently leads to higher succinicate formation. This suggests that inactivation of AK could redirect the flux from formate and acetate to the succinate producing pathway.

Effect of Deletions on Growth of *A. succinogenes* and Succinate Yields

Despite the fact that *A. succinogenes* is one of the best producers of succinate, formate and acetate are

also produced in its metabolic network in high volumes (McKinlay *et al*., 2007a). One of the metabolic engineering strategies for improving succinic acid yields is to remove competitive pathways (Liu *et al*., 2013). Phosphoenol pyruvate (PEP) is an important node in the metabolic network of *A. succinogenes* as it is the origin for directing the carbon flux towards succinate or other byproduct formation pathways. Besides PEP, oxaloacetate (OAA) seems to be another important branchpoint for succinate synthesis or transferring the flux towards pyruvate. OAA converts to succinate with malate (MAL) and fumarate (FUM) as intermediates. The yield of succinate is strongly related to NADH availability. Therefore, a flux distribution which supports higher NADH production is desirable for succinate formation (Singh *et al*., 2011; Zheng *et al*., 2013; Liu *et al*., 2010; Zheng *et al*., 2013; Li *et al*., 2010).

In this study three mutants were proposed with different knockouts in the metabolic pathways. Flux distributions for these mutants are given in Figure 2. As can be seen, the mutant 1 lacking the acetate production pathway showed the highest succinate yields among the others. For a glucose ratio of 0.5 mol.mol^{-1} substrate, a succinate yield of 0.85 mol.mol^{-1} substrate was calculated by the model for mutant1 compared to 0.64 mol.mol^{-1} substrate for the wild type strain.

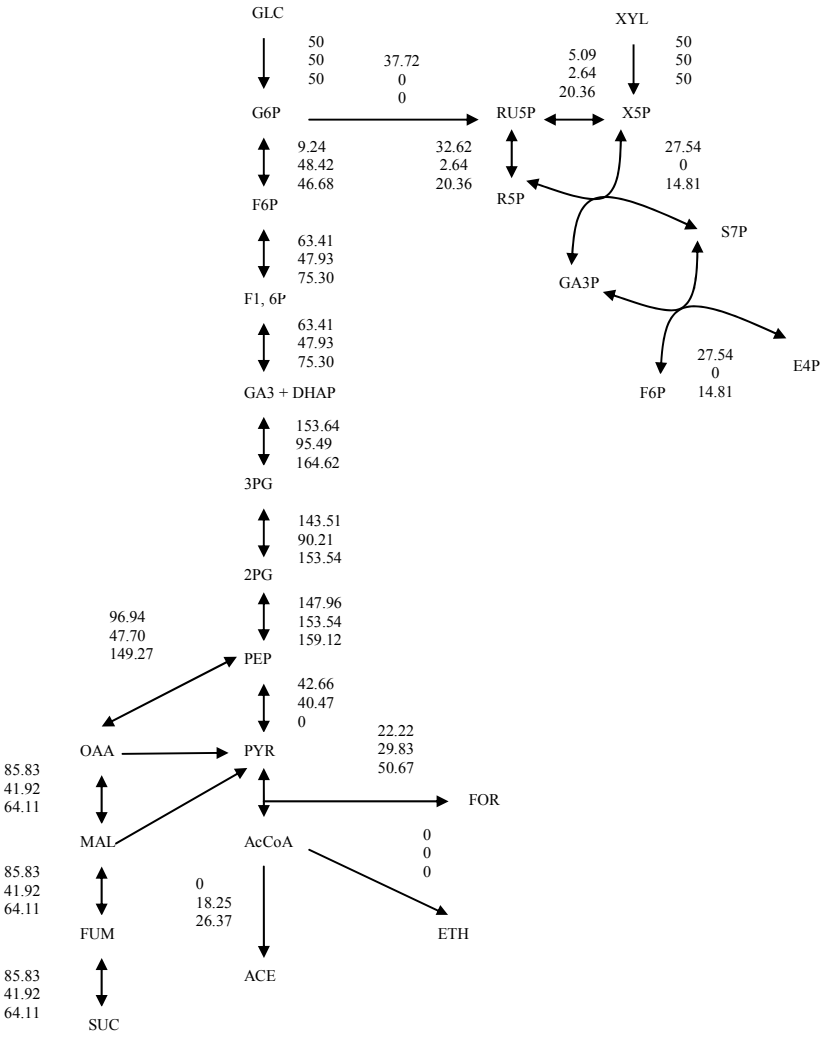

Figure 2: Metabolic fluxes of the proposed *A. succinogenes* mutants in a glucose ratio of 0.5 mol.mol^{-1} substrate. All the fluxes are calculated per 100 mol substrate.

Mutant 1 with a knockout in AcCoA→ACE +ATP
Mutant 2 with a knockout in GA3P+S7P→E4P+F6P and E4P+X5P→F6P+GA3P
Mutant 3 with a knockout in PEP↔PYR +ATP

Interestingly, the maximum growth rate and product yields for mutant3 with inactivation of puruvate kinase were similar to the wild type strain with a complete metabolic network. This is due to redirecting flux from oxaloacetate to pyruvate, while in the wild type strain the flux through this pathway was zero. The lowest succinate yield and growth rate belonged to mutant2 in which the pentose phosphate pathway was incomplete. This is reasonable as xylose degradation is through the pentose phosphate pathway and it is clear that, at lower glucose ratios, a very low growth was supported. It should be noted that increasing the glucose ratio in the substrate mixture had a positive impact on succinate yields in all the mutants.

It could be inferred from this study that inactivation of the acetic acid producing pathway in *A. succinogenes* could be offered as a good strategy for improving succinic acid yields using renewable substrates containing glucose and xylose.

CONCLUSION

Using renewable and cheap substrates for microbial processes is one of the main aims of biotechnology. Most of these sources are lignocellulosic materials which should be treated to convert them to simple sugars. In this study, the maximum capabilities of *A. succinogenes* for succinate production with glucose and xylose were studied using an FBA model. This is the first report of a metabolic model for *A. succinogenes* when the substrate is a mixture of xylose and glucose. In addition, the effect of pathway deletions on succinate production with xylose/glucose mixtures had not been studied before using metabolic models. Altogether, this study provides information about the flux distribution in *A. succinogenes* towards succinate with a mixture of xylose and glucose. In addition, it proposes genetic strategies for metabolic engineering towards strain improvement. Although the metabolic model showed a slightly lower product yield for xylose/glucose mixtures, the lower cost of the process leads to lower end product prices compared to glucose fermentation. In addition, huge amounts of agricultural wastes can be used in a dual process for simultaneous waste management and succinic acid production.

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ABBREVIATIONS

GLC	Glucose
XYL	Xylose
F6P	Fructose-6-phosphate
G6P	Glucose-6-phosphate
F1,6P	D-fructose-1,6-bisphosphate
DHAP	Dihydroxyacetone phosphate
GA3P	Glyceraldehyde 3-phosphate
1,3PG	3-Phospho-D-glyceroyl phosphate
3PG	3-Phospho-D-glycerate
2PG	2-Phospho-D-glycerate
PEP	Phosphoenolpyruvate
PYR	Pyruvate
AcCoA	Acetyl CoA
ATP	Adenosine-3-phosphate
NADH	Reduced-nicotinamide adenine dinucleotide
NADPH	Reduced-nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetate
MAL	Malate
CO2	Carbon dioxide
E4P	D-erythrose-4-phosphate
RU5P	D-Ribulose-5-phosphate
R5P	D-Ribose-5-phosphate
X5P	Xylulose-5-phosphate
S7P	D-Sedoheptulose-7-phosphate
FUM	Fumarate
SUC	Succinate
ETH	Ethanol
FOR	Formate
ACE	Acetate
Bio	Biomass

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