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Article

Influence of the initial sugar concentration and supplementation with yeast extract on the succinic acid fermentation from lactose based medium

Christiane Terboven [1], Christian Abendroth [2,3], Janin Laumer [1], Christiane Herrmann [4], Roland Schneider [4], Patrice Ramm [5], Joachim Venus [4], Matthias Plöchl [1,*]

[1] BioenergieberatungBornim (B3), Max-Eyth-Allee 101, 14469 Potsdam, Germany; ct@b3-bornim.de (C.T.); jl@b3-bornim.de (J.L.); mp@b3-bornim.de (M.P.)
[2] Institute of Waste Management and Circular Economy, Technische Universität Dresden, 01796 Pirna, Germany; christian.abendroth@tu-dresden.de (C.A.)
[3] Robert Boyle Institute e. V., Im Steinfeld 10, 07751 Jena, Germany
[4] Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB), Max-Eyth-Allee 100, 14469 Potsdam, Germany; cherrmann@atb-potsdam.de (C.H.); rschneider@atb-potsdam.de (R.S.); jvenus@atb-potsdam.de (J.V.)
[5] Institute of Agricultural and Urban Ecological Projects affiliated to Berlin Humboldt University (IASP), Philippstr. 13, 10115 Berlin, Germany; patrice.ramm@iasp.hu-berlin.de (P.R.)
* Correspondence: mp@b3-bornim.de

Abstract: The aim of this study was to investigate succinic acid production from lactose concentrate, a by-product of cheese-making, using *Actinobacillus succinogenes* and *Basfia succiniciproducens*. Although the capability of these strains to metabolize different sugars is already known, their application in the conversion of lactose bears high potential for optimization. With regard to *B. succiniciproducens* this approach is completely novel. In particular the influence of the mediums sugar concentration and its supplementation with yeast extract to prevent a lack of proteins and vitamins were examined. Lactose based media containing sugar concentrations between 20 and 65 g L^{-1} and 5 g L^{-1} yeast extract were fermented, whereby both strains showed comparable performances. The best results in succinic acid yield and acid concentration, 0.57 g g^{-1} initial sugar and 23 g L^{-1}, were achieved at an initial sugar concentration of 43 g L^{-1}. The necessity of yeast extract was demonstrated using the sugar optimized medium without supplementation. As a result, yield and concentration of succinic acid dropped to 0.34 g g^{-1} and 13 g L^{-1}, the sugar consumption decreased from more than 99 to less than 55 %. Therefore the supplementation amount of 5 g L^{-1} yeast extract can be regarded as well-balanced.

Keywords: *Actinobacillus succinogenes*; *Basfia succiniciproducens*; succinic acid; lactose concentrate; yeast extract; platform chemical

1. Introduction

Succinic acid is a volatile fatty acid of high industrial interest, especially in fine chemistry, where it can be used as a platform chemical, for example in food and pharmaceutical industries [1], or for the production of biopolymers [2]. The production of biopolymers based on succinic acid might be a good alternative to conventional plastics based on fossil oil [3].

In 2013, 38.000 t of succinic acid with a market value of $ 108 million were produced bio-based by microbial fermentation. Bio-based succinic acid is a fast-growing market with an estimated potential of $ 7 to 10 billion [3]. To satisfy the demand on bio-based succinic acid, large amounts of biomass are needed. In regard to the recent 'food versus fuel' discussion [4], organic residues and lignocellulosic biomass, representing the most abundant renewable organic sources on earth [5], should be used for the production of bio-based chemicals, such as succinic acid. In recent studies, succinic acid fermentations

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from the organic fraction of municipal solid waste [6], sugarcane bagasse [7], carob pods [8], corn stover [9], and straw [10] were investigated, using the succinogenic bacterial strains *A. succinogenes* or *B. succiniciproducens*.

There are several metabolic pathways known that are capable of succinic acid synthesis and exist in different types of microorganisms. Succinic acid is a metabolite typically occurring in all living organisms in the tricarboxylic acid cycle (TCA), where it is produced in the presence of oxygen (oxidative TCA). However, succinic acid can also be produced under anaerobic conditions utilizing the reductive TCA pathway [11]. The reductive pathway is a promising approach for succinic acid fermentation, as it allows the incorporation of carbon dioxide in degradation reactions with 6-carbon sugars [11]. Consequently, succinic acid fermentation could be used as a method to reduce the amount of industrial carbon dioxide emissions. The possibility to connect industrially produced carbon dioxide and succinic acid fermentation has been demonstrated, for example by [12] and [13].

In the past, succinic acid was synthesized by hydrogenation of maleic anhydride, a chemical produced from fossil oil [11]. In contrast, the discovery and utilization of succinic acid yielding bacteria, such as *A. succinogenes* [14] or *B. succiniciproducens* [15], allows the bio-based production of succinic acid by microbial fermentation, which is already applied on industrial scale [11].

However, in spite of the existence of industrial scale applications of succinogenic bacteria, several questions remain unsolved. Especially the fermentation of substrates which are based on different biological residues bears high potential for optimization. A promising low-cost residue for microbial fermentation is whey [16], since its applicability has already been demonstrated [17–19]. Whey can also serve for the production of protein concentrates, which are used as ingredients in sports beverages and special nutritional products [20]. The residue of the protein separation is a lactose rich whey fraction; concentrated by evaporation, this whey fraction turns into lactose concentrate. The use of lactose based substrates, such as whey or lactose concentrate, offers the advantage that the succinic acid fermentation with *A. succinogenes* is possible without pretreatment by enzymatic hydrolysis because this strain is able to utilize glucose, galactose and lactose [14]. Similar to *A. succinogenes*, *B. succiniciproducens* is capable of utilizing a diverse range of sugars, e. g. glucose, galactose, xylose, mannose and sucrose [15]. Although the fermentation of multiple sugars by *B. succiniciproducens* is described in the literature, the authors of the present study are not aware of any studies dealing with it in respect to lactose.

Besides a carbon source, bacteria need proteins and vitamins for an efficient metabolism [16]. In respect to *A. succinogenes* or *B. succiniciproducens*, an unbalanced ratio of these nutrients might decrease succinic acid productivity. Yeast extract is often used for supplementation of carbohydrate-rich media since it contains high amounts of proteins and vitamins [7,21,19]. Because yeast extract represents a significant expenditure, the reduction of its application to the minimum level is an important measure to reduce the production costs of succinic acid [22,23].

According to a modelling approach performed by [24], an optimal synthetic medium for succinic acid fermentation should contain 84.6 g L^{-1} glucose and 14.5 g L^{-1} yeast extract. Consequently, the optimal initial glucose to yeast extract ratio is 5.8. This assumption is supported by findings published by [25], who observed a limited succinic acid production at an initial glucose to yeast extract ratio of 7.5; the initial sugar concentration in the study from [25] was 75 g L^{-1}.

Usually, the succinic acid fermentation is accompanied by the synthesis of multiple by-products such as acetic acid, formic acid and lactic acid [25]. In order to decrease the synthesis of by-products and to maximize the succinic acid yield, an adaptation of the fermentation process is needed. Moreover, with regard to the production of commercial usable polybutylene succinate, the reduction of by-products is extremely profitable because monocarboxylic acids act as chain stoppers for the polymerisation [26].

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However and in particular in respect to lactose based medium, the dependencies between fermentation efficiency and nutrient composition as well as the optimal ratio of mono- and disaccharides present in the substrate, have not been discussed in detail so far and the presented work aims to close this gap.

The main objective of the present study was to improve the succinic acid productivity, using the two natural acid producers *A. succinogenes 130Z* (DSM 22257) and *B. succiniciproducens* (DSM 22022) for fermentation tests in batch mode. Moreover, and in respect to these two strains, lactose concentrate, generated from cheese whey, was used as feedstock and main compound of the fermentation media. The present study consisted of two experiments. During the first experiment the influence of the sugar concentration on the succinic acid productivity was assessed by applying sugar concentrations between 20 and 65 g L^{-1}. Concerning the low protein content of the lactose concentrate and a presumable lack of vitamins, yeast extract was used for supplementation. In the second experiment, the necessity of the supplementation with yeast extract to increase the succinic acid yields was investigated. Based on the two described experimental approaches, the present study had the following objectives:

1. Determining the potential of a by-product from cheese-making, lactose concentrate, as feedstock for bio-based succinic acid production.
2. Comparing the succinic acid fermentation performance of *A. succinogenes 130Z* (DSM 22257) and *B. succiniciproducens* (DSM 22022).
3. Examining the effect of yeast extract in lactose based medium on the succinic acid production.

2. Materials and Methods

2.1. Microbial strains and precultivation

The *A. succinogenes 130Z* (DSM 22257) and *B. succiniciproducens* (DSM 22022) were procured from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). The bacterial strains were kept as cryogenic stocks at -80°C in 50 % glycerol aqueous solution. Precultures were produced by inoculation of 100 mL tryptic soy broth in shaker flasks within 24 hours. The used orbital shaker had an agitation speed of 150 rpm. The flasks were heat sterilized at 121°C for 15 min.

2.2. Fermentation media

Lactose concentrate, a by-product from cheese-making (Karwendel-Werke-Huber GmbH & Co. KG, Buchloe, Germany), and yeast extract (Ohly KAT, Deutsche Hefewerke GmbH Nürnberg, Germany) were used for the preparation of different fermentation media. For the preparation of media, the lactose concentrate was diluted with water to adjust three different target sugar concentrations of 20, 40 and 60 ± 5 g L^{-1}, the resulting media were called A, B and C. The media for the first experiment were enriched with yeast extract in a concentration of 5 g L^{-1}. The dosage of yeast extract was indicated by the letter Y, consequently these media were called A_Y, B_Y and C_Y. Lactose concentrate and yeast extract were autoclaved separately at 121°C for 15 minutes and afterwards aseptically mixed. The second experiment was conducted with medium B, whereby the addition of yeast extract was omitted. Each fermentation was started by adding 100 mL preculture to 900 mL medium. Lactose concentrate, yeast extract and fermentation media were chemically characterized prior to their use.

2.3. Experimental setup and operation

Fermentations were carried out in batch mode utilizing a 3 L Biostat bioreactor system (Sartorius AG, Göttingen, Germany). The fermentations were conducted at a temperature of 37°C and a pH value of 6.7. The pH value was automatically regulated by the addition of 5 N NaOH. Carbon dioxide was continuously sparged into the reactor with a flow rate of 0.2 L min^{-1}. A double Rushton turbine was used for stirring with an agitation

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speed of 300 rpm. The initial volume of each fermentation batch, consisting of medium and preculture, was 1 L. The impact of the initial sugar concentration on the production of succinic acid and by-products was examined during the first experiment. Therefore, a series of fermentation tests with either *A. succinogenes* or *B. succiniciproducens* was conducted with fermentation media A_Y, B_Y and C_Y respectively.

In a second experimental approach, it was investigated if a decrease in nitrogen in the medium affects the bacterial sugar consumption and hence the acid production. Therefore, fermentations were repeated with medium B without addition of yeast extract. The test duration of the second experiment amounted to 55 hours.

During all fermentations, sampling of fermentation medium was carried out regularly in order to characterize the production kinetics of succinic acid and by-products, such as acetic, formic and lactic acid. Moreover, the amount and the composition of residual saccharides were analyzed, too. Directly upon each sampling, the succinic acid producing bacteria were inactivated by heating a sample for 20 minutes at 95°C.

2.4. Analytical methods

The concentrations of sugars and acids were obtained by high performance liquid chromatography (HPLC) using a ULTIMATE 3000 system (Thermo Fischer Scientific, Waltham, USA) with an Eurokat H column (300 mm X 8 mm X 10 µm, Knauer, Berlin, Germany) and a refractive index detector (Shodex RI-101, Showa Denko, Tokio, Japan). Peak areas and retention times were compared to standard solutions. The sugar standard solution contained glucose, galactose, fructose, arabinose and lactose. The components of the acid standard solution were succinic, acetic, formic and lactic acid. The HPLC analysis required a pretreatment of lactose concentrate, yeast extract and fermentation media. Contained sugars and acids were dissolved by a cold water extraction as described by [27]. The determination of the total Kjeldahl nitrogen content (TKN) was carried out employing a standard method [28]. The pH value was measured according to methods stated by [29, 30]. The protein content was calculated from the TKN by multiplication with the factor 6.25 [29].

2.5. Calculation of the fermentation parameters

The results retrieved from fermentation experiments were evaluated by comparing the determined final succinic acid concentration and the calculated succinic acid yield with regard to the initial sugar concentration. The formation of by-products served as additional important parameter. The synthesis of the by-products was expressed as mass ratios between succinic acid and acetic, formic or lactic acid. The total concentration of sugar was determined as sum of mono- and disaccharides and given as equivalent of glucose. The sugar consumption was defined as the amount of converted sugar in relation to the amount of initial sugar. The loss of medium due to sampling was taken into account for the calculation of the acid yields, the mass ratio between succinic acid and by-products, and the sugar consumption.

3. Results and Discussion

3.1. Feedstock and fermentation media

The chemical characteristics of the applied lactose concentrate as well as of the supplemented yeast extract, analyzed in duplicates, are given in Table 1.

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Table 1. Chemical characteristics of lactose concentrate and yeast extract.

Parameter	Lactose concentrate	Yeast extract
Sugar (g kg^{-1})[a]	268.4 ± 0.1	6.6 ± 0.2
Glucose (g kg^{-1})	7.2 ± 0.1	n.d.
Galactose (g kg^{-1})	21.2 ± 0.0	n.d.
Saccharose (g kg^{-1})[a]	n.d.	6.6 ± 0.2
Lactose (g kg^{-1})[a]	240.0 ± 0.0	n.d.
Protein (g kg^{-1})	12.0 ± 0.0	722.5 ± 13.8
Lactic acid (g kg^{-1})	37.6 ± 0.0	24.3 ± 0.7

n. d. not dectected, [a] regarded as glucose equivalents

The lactose concentrate had a sugar concentration of 268 g kg^{-1} with a lactose percentage of almost 90 % and a protein content of 12 g kg^{-1}. In comparison to the lactose concentrate, the protein content of the yeast extract was sixty times higher and had a value of 723 g kg^{-1}. The average initial sugar concentration of the media enriched with yeast extract (A_Y, B_Y and C_Y) were 21, 43 and 64 g L^{-1}, the maximum standard deviation was 1 g L^{-1}. The initial protein concentration of the media ranged between 4.5 and 6.5 g L^{-1}. About 56 to 79 % of the protein content was supplied by the yeast extract, depending on its ratio. The mass ratio of initial sugar to yeast extract of the media amounted to 4, 8 or 9 and 13 (A_Y, B_Y and C_Y). A limited succinic acid production can be expected for media showing an initial sugar to yeast extract ratio higher than 7.5, as described in the introduction. Consequently, limited succinic acid production and sugar consumption were predicted for media B_Y and C_Y.

The average initial concentration of sugar in the yeast extract free medium B amounted to 39 g L^{-1}. As for media B_Y and C_Y, a decline in succinic acid yield was also very likely for medium B. Lactose concentrate and yeast extract contained lactic acid in concentrations of 37.6 g kg^{-1} and 24.3 g kg^{-1}, shown in Table 1. Consequently, the average initial content of lactic acid in the media A_Y, B_Y, B and C_Y were 3, 6, 5 and 9 g L^{-1}.

3.2. Optimizing the sugar concentration for A. succinogenes and B. succiniciproducens

Results of the fermentation experiments with media A_Y, B_Y and C_Y are summarized in Table 2 for both strains, *A. succinogenes* and *B. succiniciproducens*. The highest yields of succinic acid were achieved with an average initial sugar concentration of 21 g L^{-1} (medium A_Y). In that case the succinic acid production was comparable for both strains, showing yields of 0.65 g (*A. succinogenes*) and 0.64 g (*B. succiniciproducens*) per g initial sugar. Application of media B_Y or C_Y resulted in small differences in succinic acid yields of both strains. *A. succinogenes* achieved 0.57 (medium B_Y) and 0.41 (medium C_Y) g succinic acid per g initial sugar, while *B. succiniciproducens* yields amounted to 0.54 (medium B_Y) and 0.33 (medium C_Y) g g^{-1}. In comparison to media A_Y, yields were 14 % and 43 % lower for media B_Y and C_Y.

Table 2. Results of the succinic acid production by *A. succinogenes* and *B. succiniciproducens* from lactose concentrate.

	A. succinogenes				*B. succiniciproducens*			
Parameter\ Medium	**A_Y**	**B_Y**	**C_Y**	**B**	**A_Y**	**B_Y**	**C_Y**	**B**
Initial sugar concentration (g L^{-1})	21	43	63	38	20	42	65	40
Initial sugar to yeast extract ratio	4	9	13	-	4	8	13	-
Fermentation duration (h)	19	46	47	55	20	46	47	54
Final succinic acid concentration (g L^{-1})	13.0	22.8	24.0	13.3	12.4	21.6	19.5	13.3
Yield of succinic acid (g g^{-1})	0.65	0.57	0.41	0.34	0.64	0.54	0.33	0.34
Residual sugar concentration (g L^{-1})	0.2	0.3	12.9	16.1	0.0	0.2	17.8	17.9
Sugar consumption (%)	99.2	99.8	75.6	53.8	100.0	99.7	68.1	50.9
Succinic acid to acetic acid ratio	1 : 0.43	1 : 40	1 : 42	1 : 0.37	1 : 35	1 : 30	1 : 32	1 : 29

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Final succinic acid concentrations of 13.0 g L^{-1} (*A. succinogenes*) and 12.4 g L^{-1} (*B. succiniciproducens*) were measured at the end of fermentation trials with medium A_Y. The increase of the average initial sugar concentration from 21 g L^{-1} (medium A_Y) to 43 g $^{-1}$ (medium B_Y) strongly enhanced the succinic acid production in respect to its final concentration, but at the expense of the yield. After 46 hours of fermentation with medium B_Y, the concentrations of succinic acid reached 22.8 g L^{-1} for *A. succinogenes* and 21.6 g L^{-1} for *B. succiniciproducens*, respectively. The course of succinic acid production during fermentation is exemplarily shown for *A. succinogenes* in Figure 1.

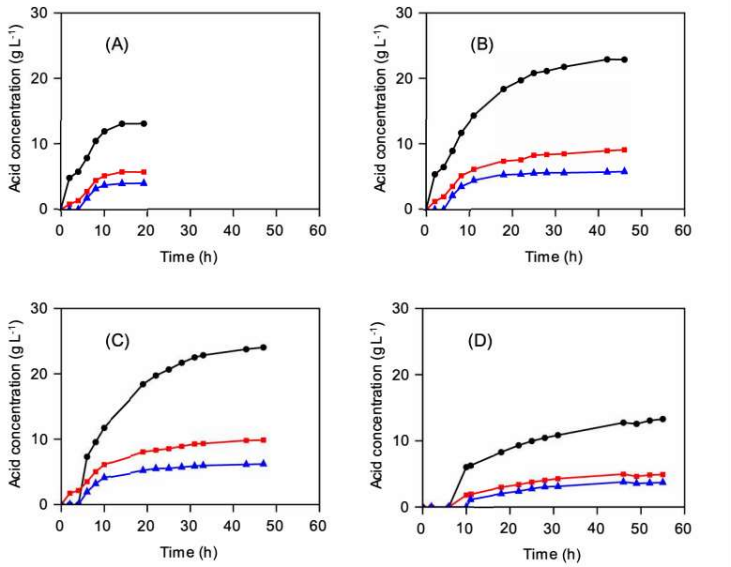

Figure 1. Acid production during fermentation with *A. succinogenes*, medium A_Y (A), medium B_Y (B), medium C_Y (C), medium B (D), concentration of succinic acid (black, circle), acetic acid (red, square) and formic acid (blue, triangle,).

Further increase of the average initial sugar concentration to 64 g L^{-1} (medium C_Y) was not effective since it caused only a small increase (*A. succinogenes*) or even a small decrease (*B. succiniciproducens*) of the succinic acid production compared to medium B_Y. This observation was accompanied by a sharp decrease of the succinic acid yield from 0.65 to 0.41 g g^{-1} initial sugar (*A. succinogenes*) and from 0.64 to 0.33 g g^{-1} initial sugar (*B. succiniciproducens*) compared to medium A_Y. Although not tested with lactose, a similar effect was observed for glucose by [25]. In the respective study, a succinic acid concentration of 41 g L^{-1} was reached by fermentation of a synthetic medium with a glucose concentration of 51 g L^{-1}, further increase of the initial glucose concentration to 74 g L^{-1} did not enhance the succinic acid production [25]. Besides negative effects on acid productivity caused by high initial sugar concentrations, a decrease of succinic acid yields might also be explained by an inhibitory effect of the acids produced during fermentation. [31] reported that the succinic acid production stopped when the concentration of the microbially produced acid mixture reached values of 45 g L^{-1}. With 49 g L^{-1} (*A. succinogenes*) and 44 g L^{-1} (*B. succiniciproducens*) in the present study, fermentation of medium C_Y resulted in concentrations, which were in a similar range as described by [31]. Therefore, the lowered yield might have occurred due to product inhibition.

With regard to the succinic acid production potential, yields observed in this study during the fermentation of lactose concentrate are in a similar range as yields from other organic by-products that are stated in literature. [7] obtained a final succinic acid concentration of 23 g L^{-1} from hydrolyzed sugarcane bagasse (supplemented with yeast extract and minerals), where the initial sugar concentration (xylose) was 52 g L^{-1}. [19]

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measured 28 g L^{-1} succinic acid after 48 hours of fermentation using a medium with an initial cheese whey concentration of 100 g L^{-1}. [6] published a techno-economic case study of the succinic acid production from the organic fraction of municipal waste and calculated profitability indicators. The calculation was based on a succinic acid productivity of 0.89 g L^{-1} h^{-1} and a production capacity higher than 40,000 t per year. From medium B_Y a similar succinic acid productivity of 0.87 g L^{-1} h^{-1} was achieved during 23-hour fermentation with *A. succinogenes*, the corresponding succinic acid yield was 0.50 g g^{-1}. *B. succiniciproducens* reached the same productivity after 20 hours of fermentation, but there was a small decline in the succinic acid yield to 0.45 g g^{-1}.

During the fermentation of media A_Y and B_Y, more than 99 % of the contained sugars were converted. When applying the medium B_Y, a decline in the sugar consumption did not occur. This is surprising, as in comparison to the results obtained with medium A_Y the conditions were less favorable in respect to initial sugar to yeast extract ratio (increase from 4 to 8 or 9).

In contrast to the efficient sugar conversion with medium B_Y, the sugar consumption decreased to less than 76 % during the fermentation of medium C_Y. At the end of the fermentation trials, the (residual) sugar concentrations in medium C_Y were 13 g L^{-1} (*A. succinogenes*) and 18 g L^{-1} (*B. succiniciproducens*) with a galactose percentage of 81 % and 80 %. Therefore, it may be assumed that both bacteria strains were capable to cleave lactose into the components glucose and galactose. Subsequently, glucose was rapidly utilized, and probably, the consumption of galactose was limited. This assumption is supported by results from previous studies. For example, while conducting succinic acid fermentations with media showing initial whey concentrations of 50 to 100 g L^{-1} and supplemented with minerals, peptone and yeast extract, [19] observed a fast consumption of glucose but a delay in the consumption of galactose, especially during the first 24 hours.

Apart from succinic acid, the main by-product of the succinic acid fermentations was acetic acid. The production of 1 g succinic acid with *A. succinogenes* from media A_Y, B_Y and C_Y was accompanied by the production of 0.42 g acetic acid on average (Table 2). In comparison to that, formic and lactic acid were formed at ratios of 0.27 g and 0.03 g per g succinic acid.

Formation of lactic acid occurred especially during the fermentation of medium C_Y with *B. succiniciproducens*. The final lactic acid concentration was 12 g L^{-1} and the final mass ratio between succinic and lactic acid reached values of 1 : 0.26. In comparison, after the fermentation of medium C_Y with *A. succinogenes* the ratio was 1 : 0.06. The fermentation of a synthetic medium containing 60 g L^{-1} glucose and 6 g L^{-1} yeast extract with *B. succiniciproducens* conducted by [9] resulted in a comparable lactic acid concentration of 10 g L^{-1}, the final mass ratio between succinic and lactic acid was 1 : 0.35.

3.3. Yeast supplementation for optimization of succinic acid fermentation

The aim of the second experiment was to find out, if the supplementation with yeast extract had a positive influence on the succinic acid production. To demonstrate the importance of nitrogen and vitamins contained in yeast extract, the experiment evaluated in section *3.2.* was repeated without adding yeast extract (medium B). A sugar concentration of about 40 g per liter was applied, because it was determined as optimal from results of the previous experiment.

The fermentations with *A. succinogenes* and *B. succiniciproducens* led to identical succinic acid yields of 0.34 g g^{-1} initial sugar (Table 2). Within 55 hours the succinic acid concentration reached a value of 13.3 g L^{-1}. Thus, the succinic acid production with medium B was about 40 % lower compared to the same medium supplemented with yeast extract (B_Y). Moreover, the sugar consumption decreased sharply from more than 99 % to 54 % (*A. succinogenes*) and 51 % (*B. succiniciproducens*), respectively.

Similar to the fermentation of medium C_Y, the degradation of galactose was limited during the fermentation of medium B. The residual sugar concentrations were 16 and 18 g L^{-1} with galactose shares of 75 % (*A. succinogenes*) and 67 % (*B. succiniciproducens*). The

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negative effect of the lack of nutrients is exemplarily shown for *A. succinogenes* in Figure 2.

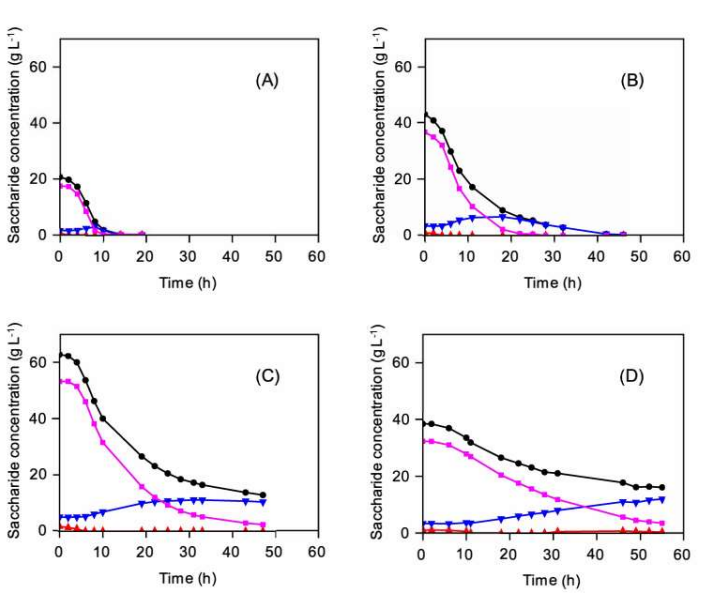

Figure 2. Sugar degradation during fermentation with *A. succinogenes*, medium A_Y (A), medium B_Y (B), medium C_Y (C), medium B (D), concentration of sugar (black, circle,), lactose (violet, square), glucose (red, upward-pointing triangle) and galactose (blue, downward-pointing triangle).

The results indicate a lack of protein or other essential nutrients, such as vitamins, that are contained in yeast extract. [32] determined a sugar consumption of only 65 % during the fermentation of diluted pineapple juice showing an initial sugar concentration of 56 g L^{-1}. [32] were able to increase the sugar consumption to 82 % due to the supplementation with 5 g L^{-1} yeast extract and minerals. The resulting initial sugar to yeast extract ratio of 11 was even higher than the optimal ratio of 5.8 derived from the model verification of [24].

4. Conclusions

Lactose concentrate from cheese production is a very suitable feedstock for succinic acid production using *A. succinogenes* or *B. succiniciproducens* as converting micro-organisms. A broad range of initial sugar concentrations from 20 to 65 g L^{-1} in the medium can be applied. Best performance was achieved at an initial sugar concentration of 43 g L^{-1} for both the acid yield per initial sugar with 0.57 g per g initial sugar and the acid concentration with up to 23 g L^{-1}. The supplementation with yeast extract appeared to be mandatory, since its absence resulted in lower succinic acid concentrations. A yeast extract concentration of 5 g L^{-1} proofed to be more than sufficient. Further investigation on the minimal concentration of yeast extract in the medium are recommended to increase the profitability of the process.

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