Utilization of nitrate abolishes the "Custers effect" in *Dekkera bruxellensis* and determines a different pattern of fermentation products.

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Abstract

Nitrate is one of the most abundant nitrogen sources in nature. Several yeast species have been shown able to assimilate nitrate and nitrite, but the metabolic pathway has been studied in very few of them. *D. bruxellensis* can use nitrate as sole nitrogen source and this metabolic characteristic can render *D. bruxellensis* able to overcome *S. cerevisiae* populations in industrial bioethanol fermentations. In order to better characterise how nitrate utilization affects carbon metabolism and the yields of the fermentation products, we investigated this trait in defined media under well controlled aerobic and anaerobic conditions. Our experiments showed that in *D. bruxellensis* utilization of nitrate determines a different pattern of fermentation products. Acetic acid, instead of ethanol, became in fact the main product of glucose metabolism under aerobic conditions. We have also demonstrated that under anaerobic conditions nitrate assimilation abolishes the "Custers effect", in this way improving its fermentative metabolism. This can offer a new strategy, besides aeration, to sustain growth and ethanol production for the employment of this yeast in industrial processes.

Keywords: *Dekkera bruxellensis*, nitrate metabolism, Custers effect, ethanol production

Introduction

Nitrate is one of the most abundant nitrogen sources in nature. In the biosphere, nitrate assimilation is the major pathway converting inorganic nitrogen to organic forms. It has been estimated that more than 2 x 10^4 megatons of organic nitrogen per year are produced by nitrate assimilation in a variety of organisms, including bacteria, fungi, algae and plants [15]**.** Several yeast species have been shown able to assimilate nitrate and nitrite, but the metabolic pathway has been studied in very few of them so far, especially in that ones which have been receiving biotechnological interest such as *Hansenula polymorpha, Pichia anomala, Pachysolen tannophilus* and *Arxula adeninivorans* [16, 2, 14, 7]. The nitrate assimilation pathway in yeast is the same as the one described for plants and filamentous fungi [29]. Two kinds of nitrate transporters have been characterized, with high and low affinity [19]. After its uptake, nitrate is converted to ammonium by two successive reductions catalyzed by nitrate reductase and nitrite reductase respectively (Fig. 1). In *H. polymorpha* the genes for nitrate transporter, nitrate reductase and nitrite reductase are clustered [3]. These genes are induced by nitrate and nitrite and repressed by ammonium as well as by other factors involved in the utilization of secondary nitrogen sources [27, 29]. This is in agreement with the preference exhibited by yeast to use inorganic compounds as nitrogen sources, like ammonium, as well as various amino acids.

Dekkera bruxellensis is often associated with wine production and lambic beer fermentation and it may contribute in a positive or negative way to the flavor development [8, 12, 21]. *D. bruxellensis* has also been reported to contaminate distilleries producing fuel ethanol, specially in continuous fermentation systems where it has been observed that this yeast can outcompete *S. cerevisiae* [18, 23]. Although *D. bruxellensis* and *S. cerevisiae* are considered as two phylogenetically very distant relatives, they share several peculiar traits, such as the ability to produce ethanol under aerobic conditions, high tolerance towards ethanol and acid, and the ability to grow without oxygen [28, 13, 17]. Apparently, these traits have evolved in parallel in both groups, but the molecular mechanisms involved may be different [28]. *D. bruxellensis* can use nitrate as sole nitrogen source [10], whereas

S. cerevisiae cannot. Woolfit et al. [32] reported the presence of five genes encoding for nitrate assimilation pathway in *D. bruxellensis*. Recently it has been shown that this metabolic characteristic can render *D. bruxellensis* able to overcome *S. cerevisiae* populations in industrial fermentations [11]. The presence of these skills together with a wide range of carbon sources utilisation by this yeast species has been leading to the idea that *D. bruxellensis* could become a new industrially relevant ethanol producing organism [4, 13, 6, 24]. In order to better characterise how nitrate utilization affects carbon metabolism and the yields of fermentation products, we investigated this trait under well controlled aerobic and anaerobic conditions and in well defined media. Our experiments showed that utilization of nitrate determines in *D. bruxellensis* a different pattern of fermentation products, in comparison to the one obtained by ammonium utilization. We have also demonstrated that nitrate assimilation abolishes the "Custers effect" under anaerobic conditions, improving its growth and fermentative metabolism.

Materials and methods

Yeast strains

The yeast strain used in this work is *Dekkera bruxellensis* CBS 2499. Stocks of the strain were stored at -80 °C in 15% v/v glycerol and revitalized prior to each experiment in liquid mineral medium (20 g l^{-1} glucose; 1.7 g l^{-1} YNB w/o amino acid and ammonium sulfate; 5 g l^{-1} ammonium sulfate).

Media and growth conditions

Aerobic batch cultivations were performed in shake-flasks and in a Biostat-Q system bioreactor (B-Braun) with a working volume of 0.8 l. The temperature was set at 30°C, the stirring speed at 500 rpm and the pH, measured by Mettler Toledo pH electrode, was adjusted at 5.0 by automatic

addition of 2M KOH. The dissolved oxygen concentration (more than 30% of air saturation) was measured by Mettler Toledo polarographic oxygen probe. The medium used was a defined synthetic medium as reported by Merico et al. [20] with the only exceptions that nitrogen sources were: ammonium sulfate, 5.0 g l^{-1}; sodium nitrate, 6.43 g l^{-1}; mixtures of ammonium sulfate, 5.0 g l^{-1} and sodium nitrate, 1 g l^{-1}, as specified. The media for anaerobic cultures were supplemented with uracil, 50 mg l^{-1}; ergosterol, 10 mg l^{-1}; and Tween 80, 420 mg l^{-1}.

The anaerobic batch cultivations were performed in Biostat-Q system. The bioreactor was flushed with nitrogen (<3 p.p.m. O_2) with a flow of 0.1 - 0.3 l l^{-1} min^{-1}. The stirring was kept constant at 500 r.p.m. Norprene tubes (Cole-Palmer, General Control, Milan, Italy) were used to minimize the diffusion of oxygen into the bioreactor. All the cultivations were repeated at least two times.

Anaerobic plate test

The plates for anaerobic test were performed on mineral medium (glucose, 20 g l^{-1}; Yeast Nitrogen Base without amino acids and ammonium sulfate, 1.7 g l^{-1}; agarose, 20 g l^{-1}) supplemented with uracil (50 mg l^{-1}), ergosterol (10 mg l^{-1}) and Tween 80 (420 mg l^{-1}) and nitrogen sources (ammonium sulfate, 5.0 g l^{-1}; sodium nitrate 6.43 g l^{-1}; mixtures of ammonium sulfate, 5.0 g l^{-1} and sodium nitrate, 1 g l^{-1}). Cells grown on liquid YPD until the exponential phase were harvested and suspended in distilled water. Approximately 500 cells were spotted on the respective plates and were grown anaerobically for two weeks. The anaerobic environment was established using Anaerocult A system (Merck, cat. no. 1138290001 and 116387) and the strength of the anaerobiosis was checked with Anaerotest strips (Merck, cat. no.115112), ensuring an oxygen content below 1 p.p.m. O_2. The assembly was prepared and the growth was followed according to Merck instructions. Each plate included the positive and negative controls, *S. cerevisiae* and *K. lactis*, respectively.

Biomass and metabolites quantification

Samples were withdrawn from the bioreactor at appropriate intervals and used to monitor the cell growth measuring the optical density at 600 nm with a spectrophotometer, after appropriate dilution. For dry weight determination, washed culture samples were filtered on a 0.45 µm glass microfiber GF/A filter (Whatman) and dried 24 h at 80°C. The concentration of extracellular metabolites, such as glucose, ethanol, acetate, nitrate and ammonium in the supernatants were determined by commercial enzymatic kits (Roche, cat. numb. 1 0716251 035, 1 0176290 035, 1 0148261 035, 1 09005658 035 and 1 1112732 035 respectively). All the assays were performed in triplicates and the standard deviations varied between 1 and 5%.

Specific consumption rates of glucose, nitrate and ammonium and specific production rates of ethanol and acetic acid were calculated during the exponential phase of growth. The yields of biomass, ethanol and acetic acid were calculated as the total amount of products divided by the total amount of glucose utilized.

Enzyme activity assays

Cell extracts were prepared by extraction with acid-washed glass beads (Sigma) according to Postma et al., [26], and the total amount of extracted proteins was quantified using the Bio-Rad kit no. 500-002 (Bio-Rad). Aceltaldeyde dehydrogenase (ACDH) and glucose 6-phosphate dehydrogenase (G6PDH) were assayed according to Postma et al., [26] with the only exception that the concentration of NADH was increased to 4 mM for the assay of NADH-dependent activity of acetaldehyde dehydrogenase. Nitrate reductase assay was performed in 50 mM potassium phosphate buffer pH 7 and 0.2 mM NADH or NADPH. The reaction was started by the addition of 10 mM sodium nitrate and the formation of NAD^+ or $NADP^+$ was followed at 340 nm. Nitrite reductase assay was performed in 50 mM potassium phosphate buffer pH 7, 10 mM $MgSO_4$, and 0.2 mM NADH or NADPH. The reaction was started by the addition of 1 mM sodium nitrite and the formation of NAD^+ or $NADP^+$ was followed at 340 nm. A unit (U) of enzyme activity is defined

as 1 μmol of substrate transformed per minute using an extinction coefficient for NAD(P)H of 6.22 l $mmol^{-1}$ cm^{-1}.

Results

Nitrate utilization under aerobic conditions

In order to obtain a detailed quantitative and qualitative analysis about the effects of utilization of nitrate on the metabolism of glucose and fermentation products in *D. bruxellensis*, batch cultures were performed in bioreactor under strictly controlled aerobic conditions, controlled pH and on synthetic media. The *D. bruxellensis* CBS 2499 strain was chosen because its genome has been sequenced [32, 25], and its glucose metabolism has been characterised under aerobic as well as under anaerobic conditions [13, 28]. In the first series of batch cultures *D. bruxellensis* was cultivated on media containing sodium nitrate as the sole nitrogen source. Under these conditions the growth rate was similar to the ammonium-based one. Interestingly, the main final product of glucose fermentation was acetic acid instead of ethanol, that reached a 3.5 times higher yield and was produced at a 2.5 times higher specific production rate than on ammonium-based ones (Table 1). On the other hand, the specific glucose consumption rate as well as the ethanol production rate were both lower on nitrate-based media than on ammonium-based ones (Table 1). The utilization of nitrate determined a slight increase of the biomass yield (Table 1). An analogous redirection of glucose catabolic products was observed also when *D. bruxellensis* was cultivated on media containing a mixture of ammonium and nitrate as nitrogen sources. In these conditions the growth rate was lower than the one observed on media containing ammonium as the sole nitrogen source (Table 1). Nitrate and ammonium were co-assimilated (Fig. 2). Also in this case the consumption of nitrate resulted in a drastic increase in the production of acetic acid, to about a three times higher

yield, and in a parallel decrease of ethanol production, to 1/3 of the yield calculated on ammonium sulfate-based media, respectively (Table 1).

Nitrate utilization under anaerobic conditions

D. bruxellensis grows under strict anaerobic condition on synthetic media at a very low rate [28]. This has been ascribed to a redox imbalance due to its scarce ability to produce glycerol, which plays an important role under anaerobic condition for the reoxidation of NADH produced during the amino acids synthesis [30]. The addition of amino acids to the medium has been shown in fact to help its growth, partially alleviating this problem [6]. Due to the dependence by the nitrate-assimilating enzymes for NAD(P)H (Fig. 1), the utilization of nitrate as nitrogen source could work in the cell as a redox sink. Nitrate metabolism has been shown to greatly facilitate growth on xylose under anaerobic conditions in the fungus *Fusarium oxysporum* [22]. To test this hypothesis, *D. bruxellensis* was cultivated under strictly controlled anaerobic conditions in bioreactor on synthetic media containing a mixture of ammonium and nitrate, due to the fact that we observed no growth on plates containing nitrate as the sole nitrogen source (not shown). Under these conditions cells grew at a higher rate than the one observed on ammonium-based medium enriched with amino acids (Table 2). The growth rate was in fact more similar to the one observed under aerobic conditions (Table 1 and 2 for comparison). In contrast to what occurred on ammonium-based media, where no acetic acid production was detected, acetic acid was produced under anaerobic cultivation when nitrate was utilized. Noteworthy, the specific acetic acid production rate corresponded to the specific nitrate consumption rate, indicating that there is a strict correlation between nitrate utilization and acetate production (Table 2). Nevertheless, in this case ethanol was the main product of glucose metabolism and its specific production rate was the highest obtained (Table 1 and 2, for comparison). Also the specific glucose consumption rate was the highest estimated, indicating that the redox unbalance negatively affected glucose metabolism and its fermentative efficiency on

ammonium-based media. All these data indicate that the assimilation of nitrate greatly improve the ability to grow under anaerobic conditions, playing like a “valve” to balance the redox potential.

Analysis of involved enzymatic activities

The activity of the enzymes involved in nitrate assimilation was assayed in *D. bruxellensis* cells growing on nitrate-based media under aerobic as well as under anaerobic conditions. Nitrate reductase was found to use either NADPH and NADH *in vitro* as the electron donor (Table 3). The activities were higher in cell extracts from anaerobic growth conditions. The activity of nitrite reductase was undetectable in cell extracts from aerobic cultures, but an extremely low activity was assayed in cells grown under anaerobic conditions, again using either NADPH and NADH as the electron donor (data not shown). In order to understand if nitrate utilization can affect the enzyme activities leading to the increased acetic acid formation, we assayed acetaldehyde dehydrogenase (ACDH). Under aerobic conditions the growth on nitrate-based media resulted in a decreased activity of ACDH (Table 3). Moreover, we observed that its affinity for NADP was higher than for NAD (see Materials and Methods). Interestingly, we found that nitrate utilization under anaerobic conditions determined an increased specific activity of NADP-dependent ACDH, which was in fact higher in nitrate-grown cell extracts than in ammonium-grown ones (Table 3) and, in parallel, a decrease in the NAD-dependent ACDH activity. On the other hand, the activity of glucose 6-phosphate dehydrogenase (G6PDH), which is one of the main sources of NADPH, was lower in nitrate-grown cells (Table 3).

Discussion

The use of nitrate as nitrogen source determines in *D. bruxellensis* deep changes in the distribution of the final fermentation products. This is well evident under aerobic and under strictly anaerobic

213 conditions. Under aerobic conditions acetic acid resulted in fact as the main product of glucose
214 metabolism, at the expense of the ethanol production (Table 1). In *D. bruxellensis* nitrate and nitrite
215 reductases can use, *in vitro*, NADH as well as NADPH as electron donors (Table 3), like most yeast
216 nitrate reductases studied so far [29]. Nitrate assimilatory enzymes and alcohol dehydrogenase
217 (ADH) can then compete for NADH, leading to a reduced ethanol synthesis. As a consequence,
218 acetaldehyde can accumulate and triggers acetic acid formation. Apparently, this is what occurs
219 under aerobic conditions. On the other hand, the stoichiometry of nitrate utilization under anaerobic
220 conditions rather suggested that nitrate reductase could mainly require, *in vivo*, NADPH as the
221 electron donor (Fig. 1). In fact the amount of acetic acid produced in this condition corresponded
222 exactly to the amount of NADPH required in the first nitrate-assimilating step, converting nitrate to
223 nitrite through a NADPH-dependent nitrate reductase (10 mmol of nitrate assimilated and 10 mmol
224 of acetic acid produced). It is noteworthy that acetic acid production has never been found under
225 strict anaerobic conditions in *D. bruxellensis* [28], being its production associated to the oxygen
226 concentration [9]. Furthermore, acetic acid specific production and nitrate specific consumption
227 showed the same rate, corroborating the strict correlation between nitrate utilization and acetate
228 production. This link can indicate that a specific need of NADPH can be satisfied by a NADP-
229 dependent ACDH activity. In agreement with this hypothesis, an increased level of NADP-
230 dependent ACDH activity was found in nitrate-grown cells in comparison to the ammonium-grown
231 cells (Table 3). Since this cofactor is required for cellular biosynthesis, in the case of nitrate
232 assimilation it could become limiting in cell metabolism, and this can in turn stimulate metabolic
233 pathways able to generate it, as acetic acid formation.
234 Another important aspect of the redox balance is linked to NAD/NADH ratio, which is an
235 especially critic step under anaerobic conditions. NADH is in fact generated not only by glycolytic
236 pathway, but also by amino acids synthesis. The most important reaction for reoxidation of this
237 surplus NADH under anaerobic conditions is the production of glycerol (Fig. 1) [30]. In *D.*
238 *bruxellensis* a very low amount of this compound was produced under those conditions [28] and the

239 inefficiency of this pathway has been indicated as the main cause of the "Custers effect" in this
240 species [31]. Nitrate assimilation could then accomplish the role of balancing the redox status. The
241 higher growth rate and higher specific ethanol production rate obtained in this work under anaerobic
242 conditions (Table 2) indicate that nitrate utilization greatly improves the fermentative metabolism in
243 *D. bruxellensis*. In *S. cerevisiae* it has been calculated that 13 mmol of glycerol per g of dry
244 biomass are generated under anaerobic conditions, leading to the reoxidation of 13 mmol of NADH
245 [1]. In *D. bruxellensis* the assimilation of 10 mmol of nitrate to ammonium through a NADH-
246 dependent nitrite reductase could result in the reoxidation of 30 mmol of NADH (Fig. 1), which fits
247 well with the theoretical formation of 26 mmol of NADH generated from the biosynthesis of 2 g of
248 dry biomass produced during its anaerobic growth.
249 In conclusion, nitrate assimilation determines in *D. bruxellensis* an improved ability to grow under
250 anaerobic conditions and enhances its fermentative metabolism, working like a redox "valve" and,
251 in this way, abolishing the "Custers effect". This offers a new strategy, besides the controlled
252 aeration, for the employment of this yeast in industrial processes.
253
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256
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341

342 Legend of Tables and Figures.

343

344 **Table 1** Growth parameters during aerobic fermentations on glucose mineral medium with
345 ammonium sulphate (5 g l^{-1}), sodium nitrate (6.43 g l^{-1}) or mixture of ammonium sulphate (5 g l^{-1})
346 and sodium nitrate (1 g l^{-1}) as nitrogen sources. **data from [28]*

347

348 **Table 2** Growth parameters during anaerobic fermentations on glucose mineral medium with
349 ammonium sulphate (5 g l^{-1}) or mixture of ammonium sulphate (5 g l^{-1}) and sodium nitrate (1 g l^{-1})
350 as nitrogen sources. **data from [28]*

351

352 **Table 3** Activity of enzymes involved in NAD(P)/NAD(P)H utilization during growth in aerobic or
353 anaerobic conditions, on glucose mineral media with ammonium sulphate (5 g l^{-1}), sodium nitrate
354 (6.43 g l^{-1}) or mixture of ammonium sulphate (5 g l^{-1}) and sodium nitrate (1 g l^{-1}) as nitrogen
355 sources. ** data from [13]*

356

357 **Fig. 1** Schematic representation of pathways involved in glucose metabolism and nitrate utilization
358 with special attention to the steps directly involved in redox balance

359

360 **Fig. 2** Batch fermentation on glucose mineral medium with a mixture of ammonium sulphate (5 g l^{-1})
361 and sodium nitrate (1 g l^{-1}) as nitrogen sources. A: substrate consumption. B: biomass and
362 metabolites production.

363

364

365

Figure
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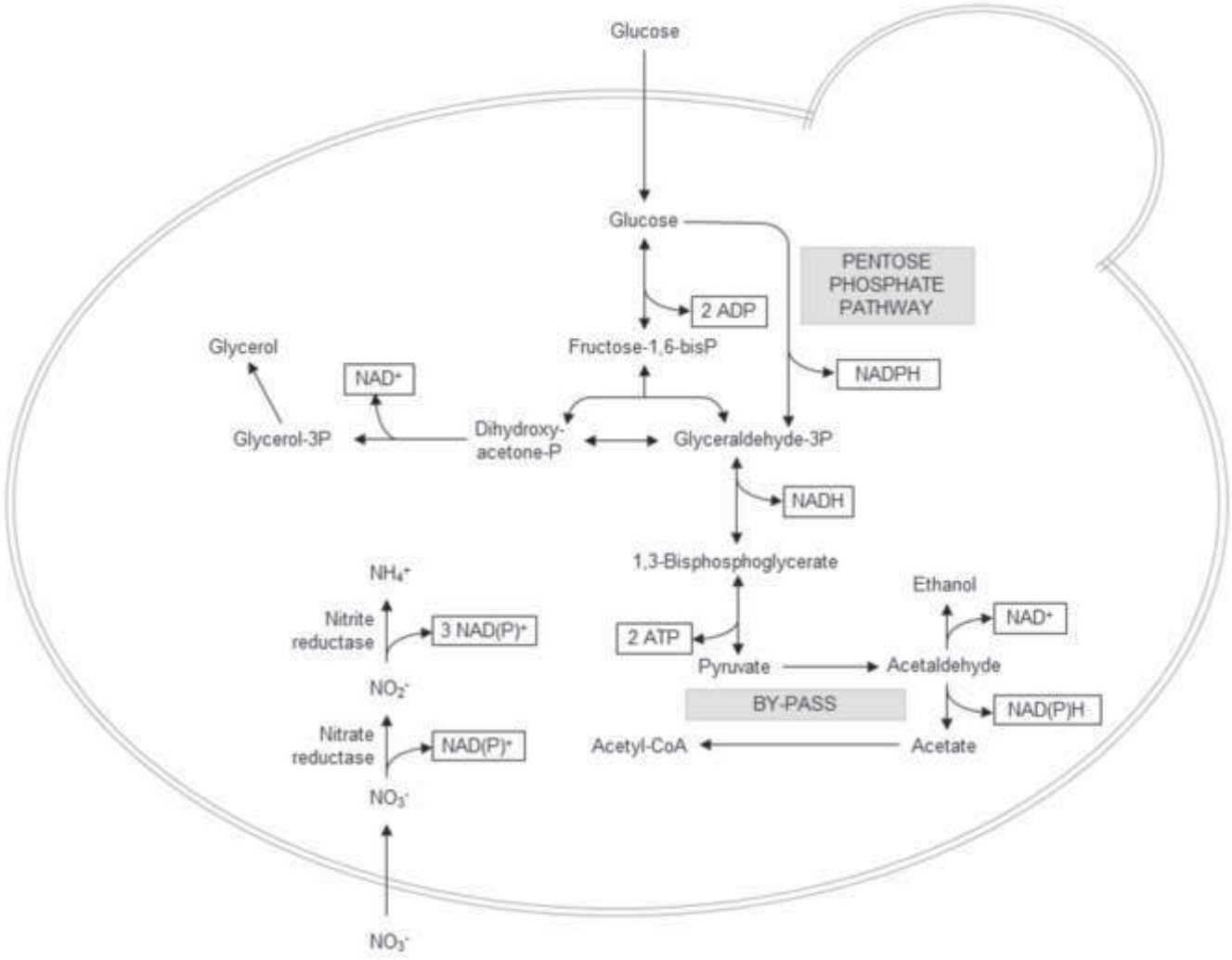

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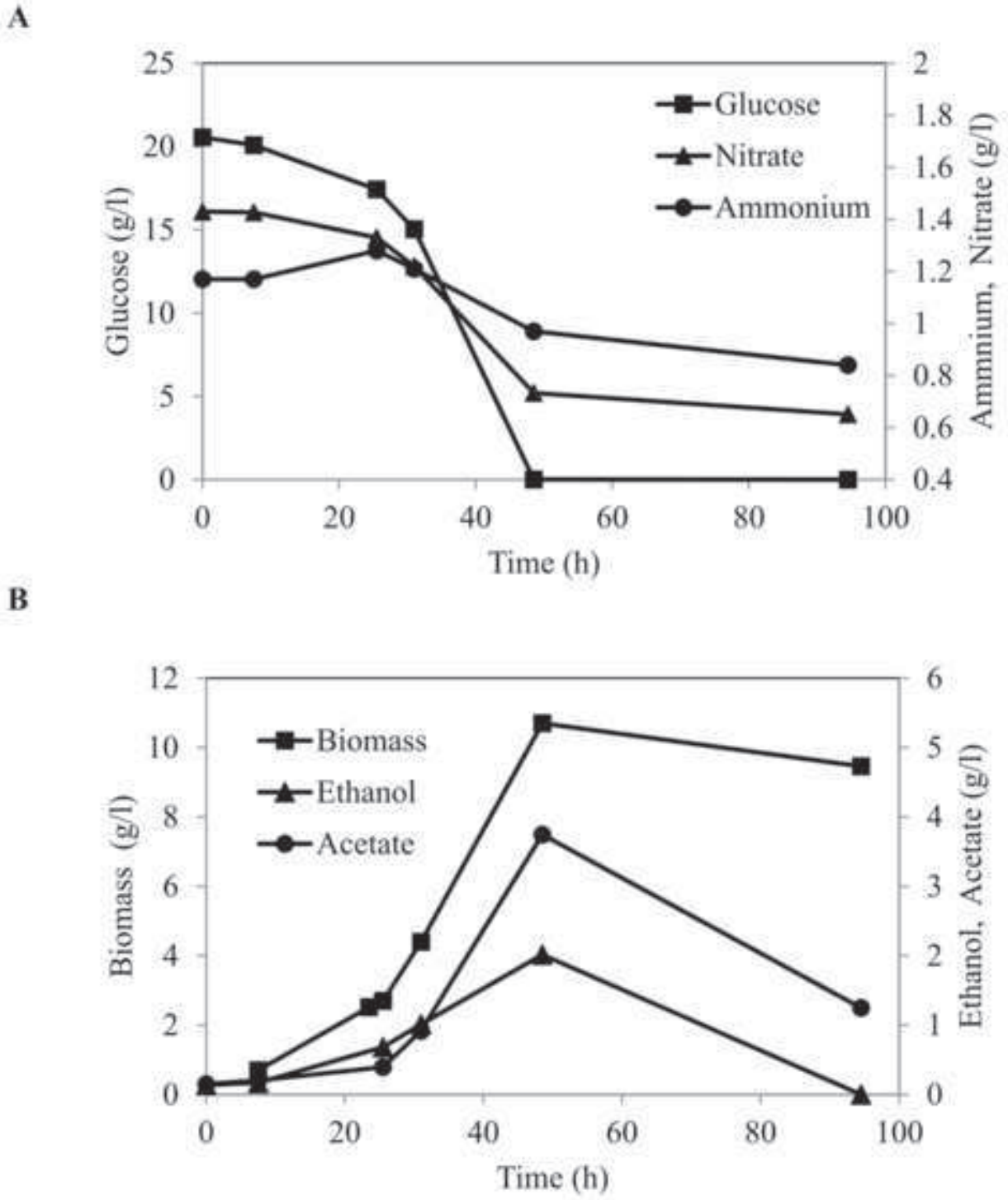

Table 1

	Ammonium*	Nitrate	Mixture
Growth rate (h^{-1})	0.11 - 0.12	0.092 ±0.006	0.077 ±0.004
q ($mMg_{dry\ weight}\ h^{-1}$)			
Glucose	3.6 - 3.7	2.94 ±0.5	2.42 ±0.28
Ethanol	3.9 - 4.4	1.65 ±0.007	1.30 ±0.09
Acetate	0.62 - 0.70	1.83 ±0.009	1.59 ±0.10
Y ($g\ g_{glucose}^{-1}$)			
Biomass	0.17 - 0.18	0.19 ±0.004	0.23 ±0.008
Ethanol	0.320 - 0.335	0.133 ±0.006	0.138 ±0.006
Acetate	0.058 - 0.060	0.216 ±0.006	0.166 ±0.011

Table 2

	Ammonium with casamino acids*	Ammonium and nitrate w/o casamino acids
Growth rate (h^{-1})	0.070 - 0.075	0.084 ±0.006
q ($mMg_{dry\ weight}\ h^{-1}$)		
Glucose	1.47 - 1.60	4.08 ±0.26
Ethanol	1.74 - 1.90	5.98 ±0.42
Acetate	0.02 - 0.02	0.43 ±0.02
Nitrate	-	0.46 ±0.02
Y ($g\ g_{glucose}^{-1}$)		
Biomass	0.132 - 0.150	0.10 ±0.005
Ethanol	0.34 - 0.35	0.35 ±0.021
Acetate	0	0.033 ±0.001

Table 3

Enzyme activity ($U\ mg_{protein}^{-1}$)	Cofactor specificity	Aerobiosis		Anaerobiosis	
		Ammonium	Nitrate	Ammonium	Ammonium and nitrate
Acetaldehyde dehydrogenase	$NADP^+$.	0.23 ±0.007	0.139 ±0.004	0.065 ±0.005	0.115 ±0.003
	NAD^+	0.50 ±0.03	0.24 ±0.014	0.13	0.025 ±0.006
Glucose 6 phosphate dehydrogenase	NADPH.	0.21 ±0.09*	0.10 ±0.007	0.16 ±0.006	0.025 ±0.003
Nitrate reductase	NADH.	-	0.039 ±0.005	-	0.045 ±0.001
	NADPH	-	0.015 ±0.002	-	0.021 ±0.001