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Efficient growth of *Kluyveromyces marxianus* biomass used as a biocatalyst in the sustainable production of ethyl acetate

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Abstract

Background: Whey is just turning from a waste of milk processing to a renewable raw material in biotechnology for producing single-cell protein, bio-ethanol, or ethyl acetate as an economic alternative. Conversion of whey-borne sugar into ethyl acetate requires yeast biomass as a biocatalyst. A high cell concentration results in a quick ester synthesis, but biomass growth means consumption of sugar at the expense of ester production. Efficient and cost-saving biomass production is thus a practical requirement. Whey is poor in nitrogen and has therefore to be supplemented with a bioavailable N source.

Methods: Several aerobic growth tests were performed with *Kluyveromyces marxianus* DSM 5422 as a potent producer of ethyl acetate in whey-borne media supplemented with various N sources. Preliminary tests were done in shake flasks while detailed studies were performed in a stirred bioreactor.

Results: Ammonium sulfate resulted in strong acidification due to remaining sulfate, but costly pH control increases the salt load, being inhibitory to yeasts and causing environmental impacts. Ammonium carbonate lessened acidification, but its supplement increased the initial pH to 7.5 and delayed growth. Urea as an alternative N source was easily assimilated by the studied yeast and avoided strong acidification (much less base was required for pH control). Urea was assimilated intracellularly rather than hydrolyzed extracellularly by urease. Conversion of urea to ammonium and usage of formed ammonium for biomass production occurred with a similar rate so that the amount of excreted ammonium was small. Ammonium hydroxide as another N source was successfully added by the pH controller during the growth of *K. marxianus* DSM 5422, but the medium had to be supplemented with some ammonium sulfate to avoid sulfur limitation and to initiate acidification. Non-limited growth resulted in 82 mg N per g of biomass, but N-limited growth diminished the N content.

Conclusions: *K. marxianus* could be efficiently produced by supplementing the whey with nitrogen. Urea and ammonia were the favored N sources due to the proton neutrality at assimilation which lessened the salt load and reduced the supply of alkali for pH control or made this even needless.

Keywords: *Kluyveromyces marxianus*; Whey; Growth; Nitrogen; Ammonium; Ammonia; Urea

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Background

Sustainable and environmentally compatible development requires successive substitution of fossil resources by renewable raw materials. This applies to the energy sector but is also true for production of industrial bulk materials. Such a bulk chemical is ethyl acetate with an annual world production of 1.7 million tons [1]. Ethyl acetate is an organic solvent of moderate polarity with versatile industrial applications. Another prospective application is the biodiesel production from vegetable oil; here, triglycerides are transformed to fatty acid ethyl esters in a lipase-catalyzed transesterification reaction with ethyl acetate as an acyl acceptor instead of methanol [2-5]. Although being an irritant and intoxicant at higher concentrations, ethyl acetate is less toxic to humans compared to many other solvents. Ethyl acetate is an environmentally friendly compound since the ester is easily degraded by bacteria [6-8] and is regarded as a non-persistent pollutant of the atmosphere [9].

Synthesis of ethyl acetate currently proceeds by petrochemical processes, which are based on crude oil constituents or natural gas, run at elevated temperature and pressure and commonly require catalysts [9]. The reactions are often incomplete, and the recovery of ethyl acetate and residual precursors needs a high input of energy.

Microbial production of ethyl acetate from renewables could become an interesting alternative. Various yeast species can synthesize ethyl acetate (reviewed by Löser et al. [9]), but only *Pichia anomala*, *Candida utilis*, and *Kluyveromyces marxianus* produce this ester in larger amounts. *K. marxianus* is the most promising candidate for large-scale ester production since this dairy yeast with GRAS status grows quickly, converts sugar directly into ethyl acetate without ethanol as an essential intermediate, and produces the ester with a high rate and yield [9-17]. The ester synthesis in *K. marxianus* is easy to control by the level of iron [11,13,16-18]. *K. marxianus* exhibits a distinct thermal tolerance which allows cultivation at an elevated temperature [14,19,20] which in turn accelerates the ester stripping and advances its process-integrated recovery. The outstanding capability of *K. marxianus* for lactose utilization offers the chance for using whey as a resource of ester synthesis. *K. marxianus* converts whey-borne lactose with a high yield into ethyl acetate [9], but its ability for sucrose and glucose assimilation [19-21] enables production of ethyl acetate with this yeast from renewables like sugarcane, grain, and corn.

Ethanol is another product of microbial sugar conversion, but several factors favor microbial ester over ethanol production [22]: the higher market price of the ester, a reduced number of process stages, a faster process, and a cost-saving product recovery.

Conversion of sugar into ethyl acetate requires yeast biomass as a biocatalyst. A high biomass concentration results in a quick process, but the production of this biomass, on the other hand, is connected with sugar consumption which reduces the portion of sugar available for ester production (as demonstrated in pilot-scale experiments [13]). The right balance between yeast growth and ester synthesis or, in other words, a compromise between a quick process and a high ester yield is of practical importance. This balance can be controlled by the available iron [13,16,23].

An efficient production of the required yeast biomass is an important factor for the economy of the total process of ester and ethanol production. At whey-based bio-ethanol production, the biomass is often considered as a gift and sugar utilization for biomass production is ignored. Such an 'out-sourcing' of biomass processing only seemingly improves the economy.

Much research was done in the field of whey-based *K. marxianus* cultivation for single-cell protein production [24-28]. The results refer to some problems at cultivation of *K. marxianus* in whey-based media; whey is poor in bioavailable nitrogen so that nitrogen can limit yeast growth [26-29]. Nitrogen-limited growth can even deregulate yeast metabolism and provoke ethanol formation at aerobic conditions [30].

Whey was often supplemented with ammonium as a source of nitrogen to stimulate growth of *K. marxianus* [10-14,16,27,29,31-37]. Added ammonium increased the yield of biomass [27,32,35] or was without significant effect [29,31,36]. Ammonium is usually supplemented in the form of ammonium sulfate where ammonium is intensively consumed while most of the sulfate remains in the medium and causes an ionic imbalance and acidification [15,38-41]. Such acidification can be inhibitory to *K. marxianus* since its growth rate is distinctly reduced at pH ≤3.5 [22,28,42,43]. Supplementation of whey with $(NH_4)_2SO_4$ or NH_4Cl requires cost-intensive pH correction with NaOH or KOH which in turn increases the salt load and thus inhibits yeast growth [31,42] and creates waste-water problems.

Urea is an alternative N source since growth with urea exhibits proton neutrality without significant pH changes [38-40]. Assimilation of urea by yeasts is not an exception but the rule; 122 of 123 tested yeasts were able to metabolize urea [44]. There are several potential advantages of urea [25,28,39]: a high amount of nitrogen per unit weight, a low price, and a reduced or even omitted supply of pH correctives. *K. marxianus* definitely metabolizes urea, and whey was repeatedly supplemented with urea as an N source for this yeast [28,31,32,37,45-47]. However, the effect of added urea on growth was often not described [37,45,46], or the published results were contradictory. Yadav et al. [28] observed an increased biomass yield, Kar and Misra [32] found no positive effect, Mahmoud and Kosikowski [31] described slight inhibition by added urea, and Rech et al. [47] even detected strong

inhibition of growth which had been attributed to alkalinization. These inconsistent results require clarification by more detailed studies on this subject.

The first objective of this work was testing the effect of $(NH_4)_2SO_4$, $(NH_4)_2CO_3$, or urea as sources of nitrogen at aerobic growth of *K. marxianus* DSM 5422 in whey-borne medium with special attention on yeast growth and acidification. When using $(NH_4)_2SO_4$, sulfate remains in the medium and causes proton imbalance and acidification but, when using $(NH_4)_2CO_3$, carbonate disappears in form of CO_2 during cultivation which possibly avoids acidification. The second objective was studying the growth of *K. marxianus* DSM 5422 in whey-borne medium with urea in detail at defined conditions to get a deeper insight into the urea metabolism. The third objective was testing the pH-controlled feed of ammonia during aerobic growth of *K. marxianus* DSM 5422 in whey-borne medium. Ammonia as the cheapest source of nitrogen is interesting for large-scale processes.

Methods

Microorganism

K. marxianus DSM 5422 from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) was maintained with the Cryoinstant preservation system (Lomb Scientific Pty Ltd, Vienna, Austria), cultivated on yeast-glucose-chloramphenicol agar (Roth GmbH, Karlsruhe, Germany) for 2 days at 32°C, and then used as an inoculum.

Non-supplemented DW medium

All media originate from concentrated and partially demineralized sweet whey. Sweet whey was ultrafiltrated, concentrated by reverse osmosis, and then demineralized by slight alkalinization and moderate heating to yield the used whey permeate (thus processed in the Sachsenmilch Leppersdorf GmbH, Leppersdorf, Germany). Non-supplemented DW medium was prepared in batches of 1 L by mixing 0.5 L whey permeate with the same volume water. This mixture was autoclaved in a sealed 1-L Schott bottle for 15 min at 121°C. Precipitated minerals were allowed to settle overnight before the upper phase was withdrawn for cultivation experiments. Non-supplemented DW medium should not be confused with DW basic medium (as used in [11-14,16]) which contains 10 g/L $(NH_4)_2SO_4$.

Shake-flask cultivation

In a first series of shake-flask experiments, various media were prepared like the just-described non-supplemented DW medium with the modification that various sources of nitrogen (10 g/L $(NH_4)_2SO_4$, 10 g/L $(NH_4)_2CO_3$, or 5 g/L urea) were added before medium sterilization. The media handling (autoclaving, settlement, withdrawal) occurred as in the above-described manner. These media were supplemented with 2 mL/L autoclaved trace-element solution (preparation as described in [10]) and diluted with water to 1/20 for reducing the content of sugar to 3.9 g/L and, thus, to avoid oxygen-limited growth at a low oxygen-transfer rate in shake flasks. Several 500-mL conical flasks with cotton plugs were filled each with 50 mL medium, inoculated with an agar-plate culture as described in [14], and shaken with 250 rpm at 32°C. After 14 h of precultivation, the process was followed by regular analysis of the optical density (OD) and the pH value over a period of at least 10 h. After a total of 40 h of cultivation, the OD and pH were measured and the residual sugar and formed biomass were analyzed.

A second series of shake-flask experiments was based on autoclaved non-supplemented DW medium. Portions of this medium were supplemented with 2 mL/L sterile trace-element solution and separately autoclaved aqueous stock solutions of $(NH_4)_2SO_4$, $(NH_4)_2CO_3$, or urea. The pH was then adjusted to 6.5 with 0.1 M HCl or KOH. These media were diluted with water to reduce the sugar content to 3.9 g/L like in the first series. Discrete sterilization of whey and N sources in this second series avoided unwanted interaction between whey constituents and N compounds such as the Maillard reaction. Inoculation, cultivation, and analyses occurred as in the first series.

Bioreactor cultivation

One reference experiment was done with DW basic medium (DW medium with 10 g/L $(NH_4)_2SO_4$) and described in detail in [11] while all other presented bioreactor processes were based on non-supplemented DW medium. The sterile 1-L stirred bioreactor with 0.6 mL Antifoam A (Fluka, Sigma Aldrich, St. Louis, USA) was filled with 0.6 L DW medium and 1.2 mL trace-element solution (the latter described in [10]). Further supplements such as $(NH_4)_2SO_4$, urea, and Na_2SO_4 were put into 100-mL Schott bottles, autoclaved for 15 min at 121°C, and then transferred to the bioreactor as follows: the bottle was connected with the bioreactor, some culture medium was pumped to the bottle, and, after complete dissolution of the substance, transported back to the reactor. Separate autoclaving avoided unwanted interaction such as the Maillard reaction and sulfate precipitation.

The cultivation occurred as usual [10-12,14]: the medium was inoculated with five loops of biomass, and the reactor was operated at 1,200 rpm and 32°C and gassed with 50 L/h dry and CO_2-free air (given for 0°C and 101,325 Pa). The pH was controlled to ≥5 with 2 M KOH or 2 M ammonia solution. All processes lasted at least 36 h. Sampling and sample preparation were performed as usual [10].

Test for urea assimilation

K. marxianus DSM 5422 was cultivated in diluted DW medium with urea (3.9 g/L sugars, 0.25 g/L urea) as described for the second series of shake-flask experiments. After depletion of the sugar, the cell suspension was separated into biomass and supernatant by centrifugation. The biomass was suspended in 50-mM phosphate buffer at pH 6 containing 0.25 g/L urea to yield an initial biomass concentration of 2 g/L, and the supernatant was supplemented with urea to give a concentration of 0.25 g/L. Both mixtures were then shaken with 250 rpm at 32°C. Sampling occurred at the beginning and after 24 h of incubation. The samples were analyzed regarding urea and ammonium.

Analyses

The optical density of cell suspensions was measured photometrically at 600 nm (after pre-dilution to OD <0.4 if required). The biomass dry weight was determined by separating the yeasts via centrifugation, washing the pellet twice, and drying at 103°C. Sugar was quantified by a modified 3,5-dinitrosalicylic-acid method [48] with lactose as a standard. Ammonium, nitrate, nitrite, sulfate, and phosphate were measured by the LCK303, LCK339, LCK342, LCK153, and LCK049 cuvette tests (Hach Lange GmbH, Düsseldorf, Germany). The total dissolved nitrogen was analyzed by the Kjeldahl method (German DIN 38409 H11). Ethanol was quantified by gas chromatography [10].

Urea was measured in the style of Rahmatullah and Boyde [49] with some modifications. This method had been approved for quantifying urea in wine [50] being a matrix similar to culture liquids. Two reagents were prepared: a mixture of 300 mL sulfuric acid (95% to 98%, ρ = 1,840 g/L), 100 mL phosphoric acid (85%, ρ = 1,670 g/L), and 100 mL water; and a solution of 500 mg butane-2,3-dione monoxime (diacetyl monoxime) and 10 mg thiosemicarbazide in 100 mL water. The analysis reagent was prepared from 40 mL acid mixture and 20 mL of the second solution and used immediately. A volume of 0.1 mL sample was mixed with 3 mL analysis reagent. In the case of colored samples, this mixture was measured photometrically at 525 nm as a blind. Then, the mixture was put in a 25-mL test tube with plastic cap and incubated for 20 min in boiling water. After cooling and short mixing, the absorbance was measured at 525 nm. The blind was subtracted, and the obtained ΔA_{525nm} value was interpreted as a urea concentration by a non-linear calibration curve prepared for several solutions of urea in water (from 0 to 500 mg/L): $C_{Urea,L} = a \cdot (\Delta A_{525nm})^b$ with a = 520 mg/L and b = 1.37.

Data analyses

The O_2 consumption, CO_2 formation, and the respiratory quotient (RQ) were calculated as usual [11]. The time-dependent specific growth rate was calculated from masses of formed CO_2 assuming a correlation between yeast growth and CO_2 formation which only applies to respiratory processes without significant maintenance: $\mu(t) \approx \Delta\ln(m_{CO2}(t))/\Delta t$. The overall biomass yield ($Y_{X/S}$) is the ratio between the mass of yeasts grown and the mass of sugar consumed; determination of these masses took losses by sampling and changes of the liquid volume into account. The pH-controlled feeding of 2 M KOH is given as a specific volume, related to the initial liquid volume of the culture. The mass of fed KOH was related to the formed biomass (given as g_{KOH}/g_X). The fed 2 M ammonia solution is also expressed as a specific volume and as the mass of supplied ammonia-N. The mass of bioavailable nitrogen was calculated as the sum of initial ammonium-N and urea-N plus the mass of ammonia-N supplied till a given time: $m_N(t) = V_L(t_0)\cdot(C_{NH_4\text{-}N}(t_0) + C_{Urea\text{-}N}(t_0)) + m_{NH_4OH\text{-}N}(t)$. The consumed nitrogen is this $m_N(t)$ value minus the nitrogen not yet used or lost by sampling: $\Delta m_N(t) = m_N(t) - V_L(t)\cdot(C_{NH_4\text{-}N}(t) + C_{Urea\text{-}N}(t)) - \Sigma m_N(\text{sampling})$. These $\Delta m_N(t)$ values were used for calculating biomass-specific consumption rates (r_N as $mg_N/g_X/h$). The overall biomass yield for nitrogen ($Y_{X/N}$) is the ratio between the mass of yeasts totally formed and the mass of N altogether consumed. The final N content of the biomass (x_N) is the reciprocal of this overall $Y_{X/N}$ value.

Results and discussion

Nitrogen in non-supplemented DW medium

The composition of whey depends on many factors such as origin of milk (cow, goat, or sheep), technology of curd production, and whey processing (e.g., [51,52]). Casein protein is coagulated by acidification (mineral or organic acids directly added or lactic acid produced in the processed milk by bacteria) and/or by using chymosin. Whey processing modifies the composition of whey as well: whey protein is separated by ultrafiltration, solutes are concentrated by reverse osmosis, and/or minerals are partially removed by alkalinization. Preparation of whey-borne culture media also changes the composition by dilution, adding supplements, and heat sterilization. This explains why published compositions of whey-borne media highly fluctuate.

Several batches of non-supplemented DW medium were analyzed regarding potential sources of nitrogen and some other parameters (Table 1). The medium is rich in \sugar and thus exhibits a high potential for biomass growth. An amount of 78 g/L sugar allows formation of 28 g/L *K. marxianus* biomass assuming $Y_{X/S}$ = 0.36 g/g (as observed at aerobic cultivation in DW basic medium with trace elements [11]). These 28 g/L biomass can only develop when yeast growth is not limited by

Table 1 Composition of non-supplemented DW medium

Parameter	Value
Sugars (88% lactose, 12% galactose)	78 g/L
Total N content (Kjeldahl analysis)	403 mg/L
Ammonium-N	106 mg/L
Urea-N	43 mg/L
Nitrate-N	4 mg/L
Nitrite-N	<1 mg/L
Organic nitrogen (except urea-N)	250 mg/L
Protein[a]	1,560 mg/L
Sulfate-S	78 mg/L
Phosphate-P	410 mg/L
Potassium	2,600 mg/L
pH value	5.7 to 6.0

Concentrated and partially demineralized sweet whey permeate was diluted with the same volume of water and autoclaved for 15 min at 121°C; precipitates were settled, and the upper clear phase was analyzed.
[a]According to the EC Nutrition Labelling Rules Directive 90/496/EEC, organic N multiplied by 6.25.

any other resource than sugar. *K. marxianus* DSM 5422 grown under such conditions exhibited the following content of minerals (in milligrams of the addressed element per gram dry biomass): $x_N = 78…79$ mg/g [10,11], $x_P = 10$ mg/g, $x_S = 4$ mg/g, and $x_K = 2$ mg/g (unpublished results). These data are similar to published compositions of *K. marxianus* [45,53-55] and *Saccharomyces cerevisiae* [40]. Multiplying these x values with the cell concentration of 28 g/L gives the required concentration of the respective element in the culture medium to allow non-limited growth: 2,200 mg/L N, 280 mg/L P, 112 mg/L S, and 56 mg/L K. Nitrogen and sulfur are lacking in non-supplemented DW medium while the phosphorous and potassium content covers the demand (Table 1).

DW medium owns 403 mg/L Kjeldahl-N (Table 1). Detailed analysis of five batches of non-supplemented DW medium gave 89 to 130 mg/L ammonium-N (106 mg/L on an average, $\sigma_{n-1} = 15$ mg/L), 38.4 to 45.9 mg/L urea-N (43 mg/L on an average, $\sigma_{n-1} = 2.6$ mg/L), 4 mg/L nitrate-N, and <1 mg/L nitrite-N. The Kjeldahl-N minus the urea-N and inorganic N gives a proteinogenic N of 250 mg/L which corresponds to ca. 1.5 g/L proteins (Table 1).

Utilization of whey proteins by *K. marxianus* is discussed controversially. Raw cheese whey owns 7 g/L protein comprising 50% β-lactoglobulin, 20% α-lactalbumin, 15% glycomacropeptide, and 15% minor protein/peptide components [51]. Their microbial hydrolysis requires excretion of proteases. Decomposition of whey proteins by *K. marxianus* has been repeatedly studied; the results varied from absent hydrolysis [26], over 20% to 33% [28,29,56], up to 80% hydrolysis [33]. Recent studies confirmed an extracellular serine protease for *K. marxianus* [57], and Yadav et al. [28] proved modification of whey proteins by *K. marxianus* via electrophoresis. Indigenous proteases in milk [58] and the proteolytic activity of lactobacilli [56] could also contribute some to protein modification during milk processing. Pre-treatment of whey protein with added proteases resulted in peptides <1 kDa which were efficiently assimilated by *K. marxianus* [59].

Some yeasts and fungi assimilate nitrate by intracellular reduction to ammonium [60]. *K. marxianus* seems to be unable for nitrate assimilation; at least several tested strains were negative [61]. This explains why nitrate added to whey did not improve growth of *K. marxianus* [32]. Some growth of *K. marxianus* in $NaNO_3$-supplemented medium was possibly caused by the added yeast extract [62]. *K. marxianus* DSM 5422 proved to be unable to assimilate nitrate.

Due to the uncertainty of whey-protein assimilation by *K. marxianus*, it is assumed that whey proteins do not contribute to assimilable nitrogen. The utilizable nitrogen in non-supplemented DW medium of ca. 150 mg/L (ammonium-N plus urea-N) is thus much smaller than the calculated demand. Supplementation of DW medium with nitrogen is essentially required.

K. marxianus DSM 5422 grows well with ammonium [10,11], but its growth with urea has not yet been tested. Yeasts generally assimilate urea [44] which should also apply to the studied strain.

A low amount of iron, zinc, and copper in DW medium limits growth of *K. marxianus* DSM 5422 [11]. Here, the medium was ever supplemented with trace-element solution to avoid such limitation.

Test of several sources of nitrogen without previous pH adjustment

This preliminary test for assimilation of diverse sources of nitrogen by *K. marxianus* DSM 5422 was performed in shake flasks. Non-supplemented DW medium was spiked with several N sources (Table 2): no supplement as a reference, $(NH_4)_2SO_4$ as the usual N supplement, $(NH_4)_2CO_3$ as an alternative ammonium resource, and urea as another N compound. The DW medium contributed ca. 7 mg/L N (5 mg/L NH_4-N and 2 mg/L urea-N) to the assimilable N in each culture. The initial pH value was influenced by the added N source: $(NH_4)_2SO_4$ did not change the pH while urea and $(NH_4)_2CO_3$ alkalinized the medium (Table 2). All shake flasks were inoculated and pre-cultivated for 14 h before the process was followed by repeated OD and pH measurements (Figure 1).

The process without a supplement was at first similar to the process with $(NH_4)_2SO_4$ (Figure 1), but later, nitrogen became a limiting factor so that growth and medium acidification slowed down and nearly stopped after 24 h. The N-limited growth resulted in some residual sugar (Table 2).

Table 2 Parameters at aerobic growth of *K. marxianus* DSM 5422 in media with various sources of nitrogen without previous pH adjustment

Parameter		Added source of nitrogen			
		No supplement	$(NH_4)_2SO_4$	$(NH_4)_2CO_3$	Urea
Supplied N	[mg/L]	0	106	146	117
NH_4-N + urea-N	[mg/L]	7	113	153	124
Initial pH value	[–]	5.96	5.94	7.50	7.18
μ at t = 14 h	[h^{-1}]	0.51	0.57	0.29	0.57
Final sugar	[g/L]	0.92	0.00	0.15	0.00
Final biomass	[g/L]	0.47	1.23	1.06	1.29
Final pH value	[–]	3.99	2.47	3.45	7.10

The whey and the sources of nitrogen were autoclaved together; the data belong to the experiment shown in Figure 1; the final parameters were measured after a total of 40 h of cultivation.

With a supplement of $(NH_4)_2SO_4$, *K. marxianus* DSM 5422 grew at first with $\mu = 0.57\ h^{-1}$ as usual in diluted DW medium [12,14], but then, the growth slowed down due to medium acidification (Figure 1). A pH <3.5 impairs growth of *K. marxianus* [28,42,43]. This acidification was caused by consumption of ammonium without an equivalent uptake of sulfate. The use of ammonium sulfate as an N source calls for buffered medium (in shake-flask experiments [12,14]) or for pH control (in bioreactor experiments [10,11,13,16,22]).

The supplement of $(NH_4)_2CO_3$ at first alkalinized the medium. The initial pH of 7.5 impacted growth of *K. marxianus* DSM 5422 and caused a highly retarded process (Figure 1). Vivier et al. [42] and Antoce et al. [43] found that a pH >7 is adverse for growth of *K. marxianus*. Continued growth gradually reduced the pH and accelerated yeast growth so that all sugar had been consumed after 40 h. Carbonate as an exchangeable anion can leave the medium in the form of carbon dioxide which counteracted acidification (medium with $(NH_4)_2CO_3$) while sulfate as a permanent ion remains and resulted in unfavorable acidification (medium with $(NH_4)_2SO_4$).

The supplement of urea also alkalinized the medium to some degree (initial pH = 7.18) which is, at the first view, surprising since urea reacts neutral in aqueous solution. Autoclaving the medium together with urea maybe caused some hydrolysis of urea to form alkaline ammonium carbonate. This slight alkalinization slowed down yeast growth and retarded the process a little at the beginning, but the yeast metabolism lowered the pH so that the growth rate approached a normal value during pre-cultivation (Table 2). After 21 h, the medium acidification stopped and then the pH increased to a final pH of 7.1 (i.e., only temporary decrease in pH). Absence of enduring pH changes confirms the earlier postulated proton neutrality at growth with urea [38-40].

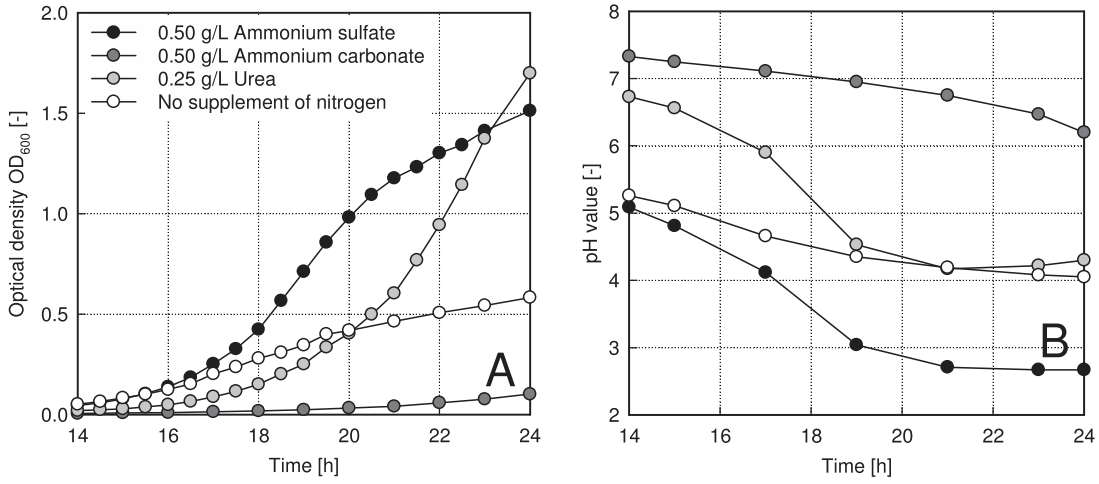

Figure 1 Aerobic batch cultivation of *K. marxianus* DSM 5422 without pH adjustment. Aerobic batch cultivation of *K. marxianus* DSM 5422 in media with various sources of nitrogen without previous pH adjustment. Concentrated and partially demineralized sweet whey was diluted with the same volume of water, supplemented with various sources of nitrogen, and autoclaved; the clear upper phase was supplemented with trace elements and diluted again with water resulting in media with 3.9 g/L sugar; the media were inoculated and cultivated in conical flasks at 250 rpm and 32°C.

Test of several sources of nitrogen with previous pH adjustment

The just-described shake-flask experiments were repeated with two modifications: all sources of nitrogen were autoclaved separately to eliminate unwanted interaction between the N sources and medium constituents, and the initial pH value was adjusted to 6.5 for avoiding alkaline conditions.

The convenient initial pH of 6.5 produced a uniformly high specific growth rate of $\mu = 0.59\ h^{-1}$ (Table 3) resulting in very similar cell densities at the beginning of the observation period (Figure 2A). This μ value was identical with the growth rate of *K. marxianus* DSM 5422 in highly diluted and phosphate-buffered DW basic medium [12,14].

The culture without a supplement of nitrogen exhibited N-limited growth (Figure 2A), only moderate acidification owing to restricted growth (Figure 2B), a low biomass formation, and some residual sugar after 40 h of cultivation (Table 3). Supplementation with $(NH_4)_2SO_4$ or $(NH_4)_2CO_3$ resulted in a nearly identical growth behavior and similar final pH values and biomass yields (Table 2); the pH was suboptimal in both cases which caused impaired growth compared to the process in phosphate-buffered medium [12,14]. A reduced biomass yield for *K. marxianus* in whey at a low pH has also been described by Yadav et al. [28] and interpreted as diversion of lactose from anabolism (growth) toward catabolism (maintenance). The use of $(NH_4)_2CO_3$ instead of $(NH_4)_2SO_4$ was without advantage since the initial pH adjustment of $(NH_4)_2CO_3$-supplemented medium with hydrochloric acid caused substitution of exchangeable by permanent ions (carbonate replaced by chloride). $(NH_4)_2CO_3$ is thus not a useful alternative since a high initial pH inhibits yeast growth while preceding pH adjustment with acids eliminates the buffering effect of carbonate.

Separate autoclaving of the N source and initial pH adjustment caused good growth in the urea-supplemented medium from the beginning; then, the growth became somewhat retarded (at 18 to 22 h; Figure 2A), but afterward, the growth accelerated again (at t >22 h) and even exceeded growth with $(NH_4)_2SO_4$ (Table 3). Quick growth in the first period was possibly based on whey-borne ammonium while the temporal slowdown of growth in the second period perhaps came about through the adaptation of yeast metabolism to urea assimilation after NH_4-N depletion. The quite high biomass yield with urea is certainly attributed to an appropriate pH value over the whole growth period (compare [39,62]). Urea is thus a promising source of nitrogen for *K. marxianus*. The acidification with urea was only of temporal nature; the pH increased again to give a final value being nearly identical with the initial pH (due to proton neutrality at assimilation of urea [38-40]).

Assimilation of urea by *K. marxianus* DSM 5422

Ammonium is utilized by all common yeasts directly while urea is either hydrolyzed by urease to form ammonium or it is assimilated via the urea amydolyase pathway [25,38]. Urease acts extracellularly while the amydolyase pathway works intracellularly. *K. marxianus* is regarded as a urease-negative yeast [40] and should metabolize urea only in the latter way being a two-step process [44,60,63]; urea reacts with hydrocarbonate in an energy-consuming process to form allophanate which, in turn, is hydrolyzed to release ammonium:

$$\mathrm{NH_2\text{-}CO\text{-}NH_2 + HCO_3^- + ATP \rightarrow NH_2\text{-}CO\text{-}NH\text{-}COO^- + H_2O + ADP + P_i} \quad (1)$$

$$\mathrm{NH_2\text{-}CO\text{-}NH\text{-}COO^- + 3\ H_2O + H^+ \rightarrow 2\ NH_4^+ + 2\ HCO_3^-} \quad (2)$$

These two reactions are catalyzed by urea carboxylase and allophanate hydrolase [44,60,63]. The produced ammonium is then metabolized in the same manner as ammonium taken up directly.

Table 3 Parameters at aerobic growth of *K. marxianus* DSM 5422 in media with various sources of nitrogen with initial pH adjustment

Parameter		Added source of nitrogen			
		No supplement	$(NH_4)_2SO_4$	$(NH_4)_2CO_3$	Urea
Supplied N	[mg/L]	0	106	146	117
NH_4-N + urea-N	[mg/L]	7	113	153	124
Initial pH value	[–]	6.50	6.50	6.50	6.50
μ at t = 14 h	[h^{-1}]	0.59	0.59	0.59	0.59
Final sugar	[g/L]	1.03	0.00	0.00	0.00
Final biomass	[g/L]	0.47	1.29	1.25	1.44
Final pH value	[–]	4.29	2.43	2.54	6.45

The whey and the sources of nitrogen were autoclaved separately; the data belong to the experiment shown in Figure 2; the final parameters were measured after a total of 40 h of cultivation.

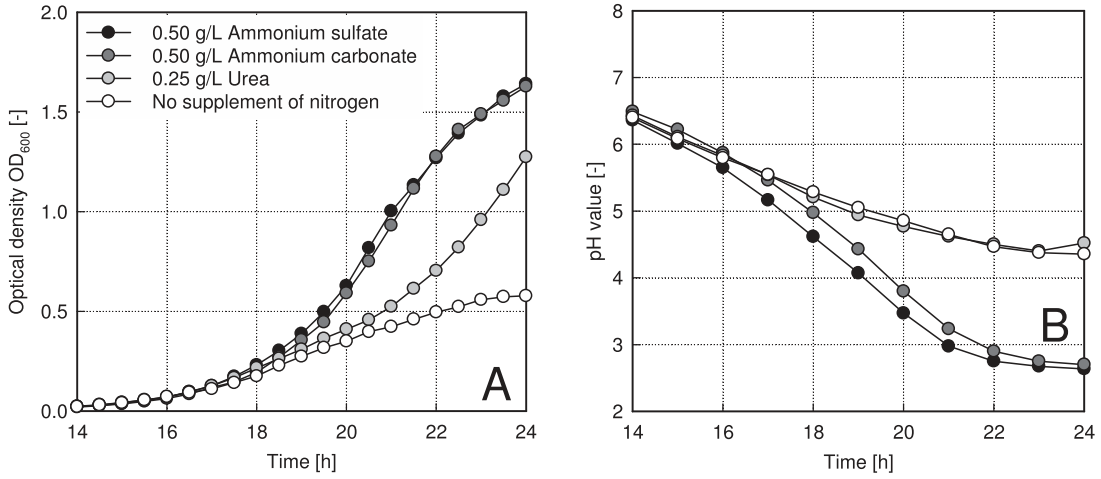

Figure 2 Aerobic batch cultivation of *K. marxianus* DSM 5422 with initial pH adjustment. Aerobic batch cultivation of *K. marxianus* DSM 5422 in media with various sources of nitrogen with initial pH adjustment. Non-supplemented DW medium was supplemented with various sources of nitrogen and trace elements, the pH was adjusted to 6.5 by HCl or KOH, and these media were diluted with water resulting in 3.9 g/L sugar; the media were inoculated and cultivated in conical flasks at 250 rpm and 32°C.

An experiment was done for clarifying the way of urea assimilation in *K. marxianus* DSM 5422. The yeast was cultivated in diluted DW medium with urea, and the obtained culture was then used in an above-described test. *K. marxianus* DSM 5422 did not excrete urease into the medium since urea added to the cell-free aqueous fraction of this culture was not hydrolyzed at all. This is in accordance with Nahvi and Moeini [61] who found all tested *K. marxianus* and *K. lactis* strains being urease negative. These findings argue for assimilation of urea via the amydolyase pathway. The grown yeast biomass was incubated with urea in a phosphate buffer but only a bit urea was reacted to ammonium (average reaction rate 0.25 mg urea/g_X/h). Absent sugar obviously suppressed transfer of urea into ammonium which refers to an amydolyase pathway being under transcriptive control.

Bioreactor cultivation with the addition of urea

The shake-flask experiments clearly demonstrated the capability of *K. marxianus* DSM 5422 for urea assimilation. Urea is a promising source of nitrogen due to the proton neutrality at its consumption during yeast growth [38-40], avoiding strong acidification as happening at growth with $(NH_4)_2SO_4$. Urea has been repeatedly used at growth of *K. marxianus*, but the obtained results were inconsistent [28,31,41,47,62]. Hensing et al. [39] referred to the potential risk of an imbalance between release and assimilation of ammonium; the medium could alkalinize when ammonium is quicker released from urea than is incorporated into biomass. Such an alkalinization was observed during cultivation of *K. marxianus* in urea-supplemented whey [47]. In the above-described experiments, the pH temporally decreased rather than increased. This phenomenon has not yet been understood and requires clarification in a bioreactor experiment.

K. marxianus DSM 5422 was cultivated in a stirred reactor at well-defined conditions (32°C, pO_2 ≥30% air saturation, pH ≥5) in DW medium which was supplemented with urea, Na_2SO_4, and trace-element solution to avoid limitation of growth by nitrogen, sulfur or microelements. Another bioreactor experiment performed with DW medium containing 10 g/L $(NH_4)_2SO_4$ and trace elements was taken from [11] and used here as a reference. These two processes are depicted in Figure 3, and characteristic parameters are summarized in Table 4.

The courses of yeast growth, sugar consumption, and marginal ethanol formation were very similar for $(NH_4)_2SO_4$ or urea as the added N sources (Figure 3A). The only marked difference was the amount of formed biomass which was apparently higher with urea (Table 4). This observation is in accordance with Hensing et al. [39] and Rajoka et al. [62]. From the energetic point of view, growth with urea should be less effective compared to growth with ammonium since assimilation of urea via the amydolyase pathway requires ATP [44,60,63]. Cultivation with urea should therefore result in a lower rather than a higher biomass yield. In case of Hensing et al. [39] and Rajoka et al. [62], the observed low growth with ammonium was possibly caused by inhibitory acidification due to absent pH control at shake-flask cultivation. Such an inhibitory acidification was prevented by controlling the pH during bioreactor cultivation (Figure 3B); here, the diverging biomass yields were presumably caused by slightly different amounts of bioavailable nitrogen (1.49 g N in medium with 5 g/L urea, and 1.30 g N in medium with 10 g/L $(NH_4)_2SO_4$). Nearly all bioavailable nitrogen was assimilated in both processes (only 20 or 30 mg/L residual NH_4-N in the culture broth; Table 4) which refers to a

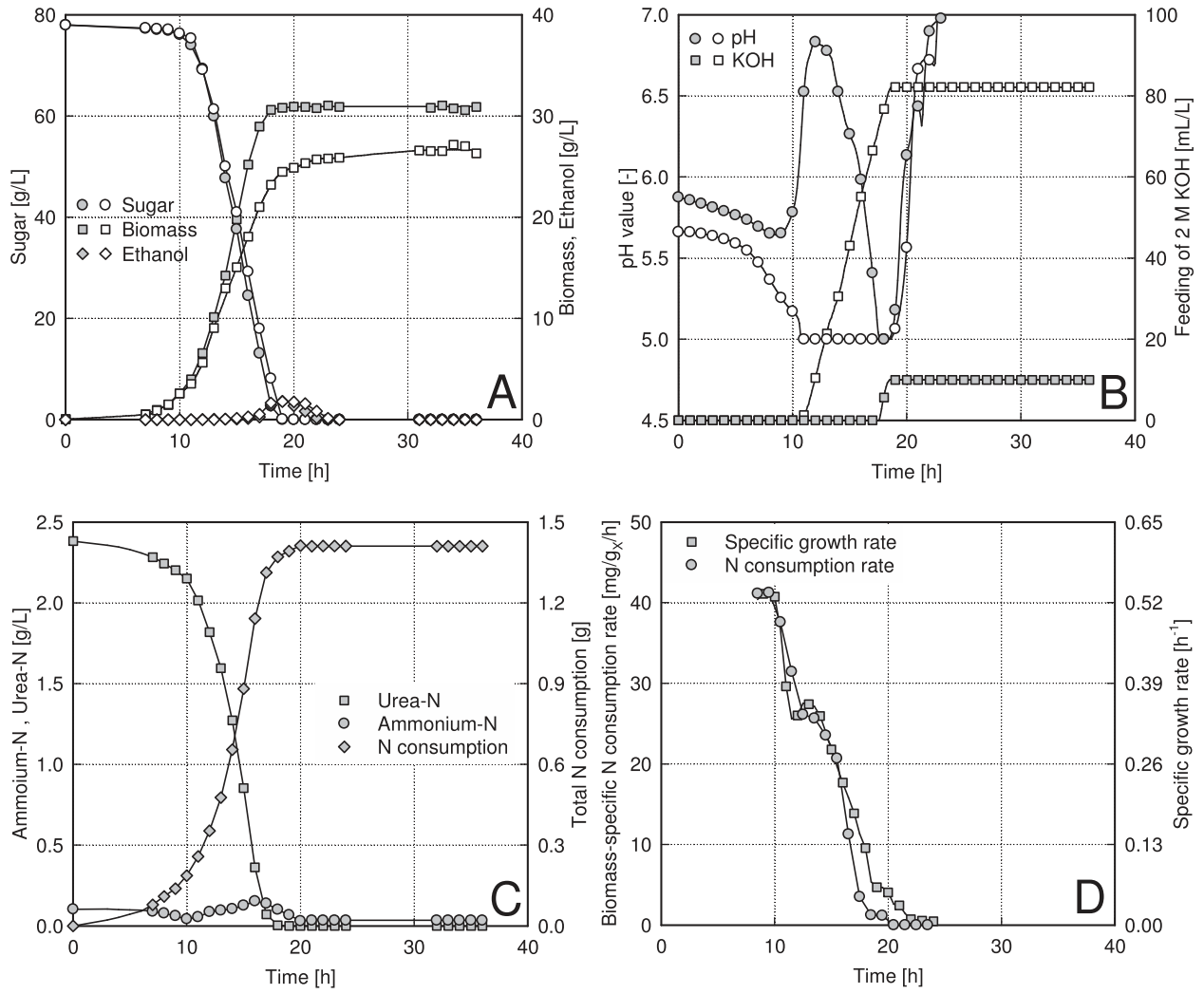

Figure 3 Aerobic batch cultivation of *K. marxianus* DSM 5422 in stirred bioreactors using ammonium or urea. Aerobic batch cultivation of *K. marxianus* DSM 5422 in a stirred bioreactor using ammonium (white symbols) or urea (grey symbols) as a source of nitrogen. DW medium with 2 mL/L trace-element solution was supplemented with $(NH_4)_2SO_4$ or urea plus Na_2SO_4 (white symbols = 10 g/L $(NH_4)_2SO_4$; grey symbols = 5 g/L urea and 0.4 g/L Na_2SO_4); the cultivation occurred in an 1-L stirred reactor at 1,200 rpm, 32°C, and aeration with 50 L/h; the pH was controlled to ≥5 with 2 M KOH; the given growth rate was derived from measured CO_2 data.

slight deficit of nitrogen, and this deficit was more striking with $(NH_4)_2SO_4$. This argumentation is supported by a higher N content in the biomass grown with urea (Table 4).

In urea-supplemented medium, *K. marxianus* DSM 5422 grew at first quickly; but later, the growth slowed down more and more (Figure 3D), although sufficient O, N, P, S, K, and trace elements should allow non-limited growth over an extended period. This behavior could be explained with a lack of vitamins in whey [27,29] or with inhibition by whey-borne minerals [31]. Supplementing whey with yeast extract [27,29] or vitamins [29,32,47] stimulated the growth of *K. marxianus*.

The source of nitrogen distinctly influenced the pH(*t*) course (Figure 3B). With $(NH_4)_2SO_4$, the pH decreased due to ammonium consumption until the pH was controlled to pH 5; 82 mL/L 2 M KOH were supplied which corresponds to a specific dosage of 0.34 g KOH per g of produced biomass. The strong acidification with $(NH_4)_2SO_4$ is explained by a proton imbalance [38-40]: ammonium was consumed while most of the sulfate remained in the medium. With urea, the pH at first a little decreased, then sharply rose to pH 6.8 and reduced again to pH 5 where the pH controller avoided further acidification; the KOH dosage was in fact much smaller (only 10 mL/L 2 M KOH or 0.04 g KOH per g of produced biomass; Table 4). Hensing et al. [39] cultivated *K. lactis* in galactose medium with $(NH_4)_2SO_4$ or urea; the acidification was strong and permanent with $(NH_4)_2SO_4$, while the acidification was moderate and only temporary with urea.

During cultivation of *K. marxianus* DSM 5422 in urea-supplemented DW medium, the dissolved ammonium-N

Table 4 Parameters at aerobic growth of *K. marxianus* DSM 5422 in media with various sources of nitrogen in a stirred bioreactor

Parameter		Added sources of nitrogen…				
		$(NH_4)_2SO_4$ [a]	Urea	NH_4OH	$(NH_4)_2SO_4$ + NH_4OH	$(NH_4)_2SO_4$ + NH_4OH
Added $(NH_4)_2SO_4$	[g/L]	10	0	0	1	2
Added urea	[g/L]	0	5	0	0	0
Added Na_2SO_4	[g/L]	0	0.4	0.4	0	0
Initial NH_4-N content	[g/L]	2.12	0.10	0.13	0.30	0.53
Initial urea-N content	[g/L]	0.04	2.38	0.04	0.04	0.04
Maximum growth rate	[h^{-1}]	0.56	0.54	0.54	0.54	0.56
Consumed sugar	[g]	45.2	44.7	44.3	44.8	44.9
Final cell concentration	[g/L]	26.7	30.8	7.49	22.1	27.0
Formed biomass	[g]	16.3	17.3	4.2	13.0	16.2
Overall $Y_{X/S}$	[g/g]	0.362	0.388	0.095	0.289	0.361
Final NH_4-N content	[g/L]	0.02	0.03	0.00	0.04	0.17
Final urea-N content	[g/L]	0.00	0.00	0.00	0.00	0.00
Supplied 2 M NH_4OH	[mL]	0.0	0.0	0.0	24.8	39.8
Supplied ammonia-N	[g]	0.000	0.000	0.000	0.694	1.114
Consumed NH_4-N	[g]	1.212	0.039	0.077	0.849	1.317
Consumed urea-N	[g]	0.024	1.371	0.025	0.026	0.023
Consumed N	[g]	1.236	1.410	0.102	0.875	1.340
Overall $Y_{X/N}$	[g/g]	13.2	12.3	41.2	14.9	12.1
Final N content, x_N	[mg/g]	76	81	24	67	83
Supplied 2 M KOH	[mL/L]	82	10	0	0	0
Supplied KOH	[g_{KOH}/g_X]	0.34	0.04	0.00	0.00	0.00
Consumed oxygen	[g]	25.3	26.4	28.6	30.3	27.9
Formed CO_2	[g]	35.1	37.2	41.2	42.5	39.0
Average RQ	[mol/mol]	1.01	1.02	1.05	1.02	1.02
Maximum $C_{EtOH,L}$	[g/L]	1.8	1.8	<0.1	0.2	<0.1

Final parameters are valid for 36 h of cultivation with exception of the process with NH_4OH as the only added N source where the final parameters were determined for 40 h of cultivation; cultivation at conditions as stated in Figures 3 and 4.
[a]Data in this column was in part taken from Urit et al. [10,11,14]; small deviations from the formerly published data are due to re-evaluation of basic data taking liquid sampling into account.

and urea-N were repeatedly measured and used for calculating the N consumption (Figure 3C). Intracellular conversion of urea to ammonium and usage of this ammonium for biomass growth occurred with nearly the same rate since ammonium excretion was only marginal (some NH_4-N originated from the used whey). The N consumption correlated well with the yeast growth (compare Figure 3A and C) and, thus, the courses of the N consumption rate and the specific growth rate were similar (Figure 3D). The quotient of these rates represents a momentary $Y_{X/N}$ value.

The small transient NH_4-N accumulation (Figure 3C) partially correlated with the temporary increase in pH. Hensing et al. [39] already referred to the danger of alkalinization when ammonium release exceeds ammonium assimilation. Such an alkalization to pH 8.5 was observed by Rech et al. [47] at cultivation of *K. marxianus* in urea-supplemented whey causing severe growth inhibition. Here, such an inhibition did not occur (only moderate rise of pH to 6.8).

Bioreactor cultivation at a pH-controlled feed of ammonia

Feeding the required nitrogen in form of ammonia could be a cost-saving alternative. Ammonia was repeatedly used as an N source at cultivation of *K. marxianus* in whey or other media [34,64-67] but dissolved ammonium or N consumption has not been paid much attention, with exception of Hack and Marchant [65] who depicted the time-dependent supply of ammonia.

In another series of bioreactor experiments, *K. marxianus* DSM 5422 was cultivated in DW medium as before but 2 M NH_4OH was used as the predominating N source which was supplied by the pH controller at pH <5. Three experiments were performed with a varied mass of

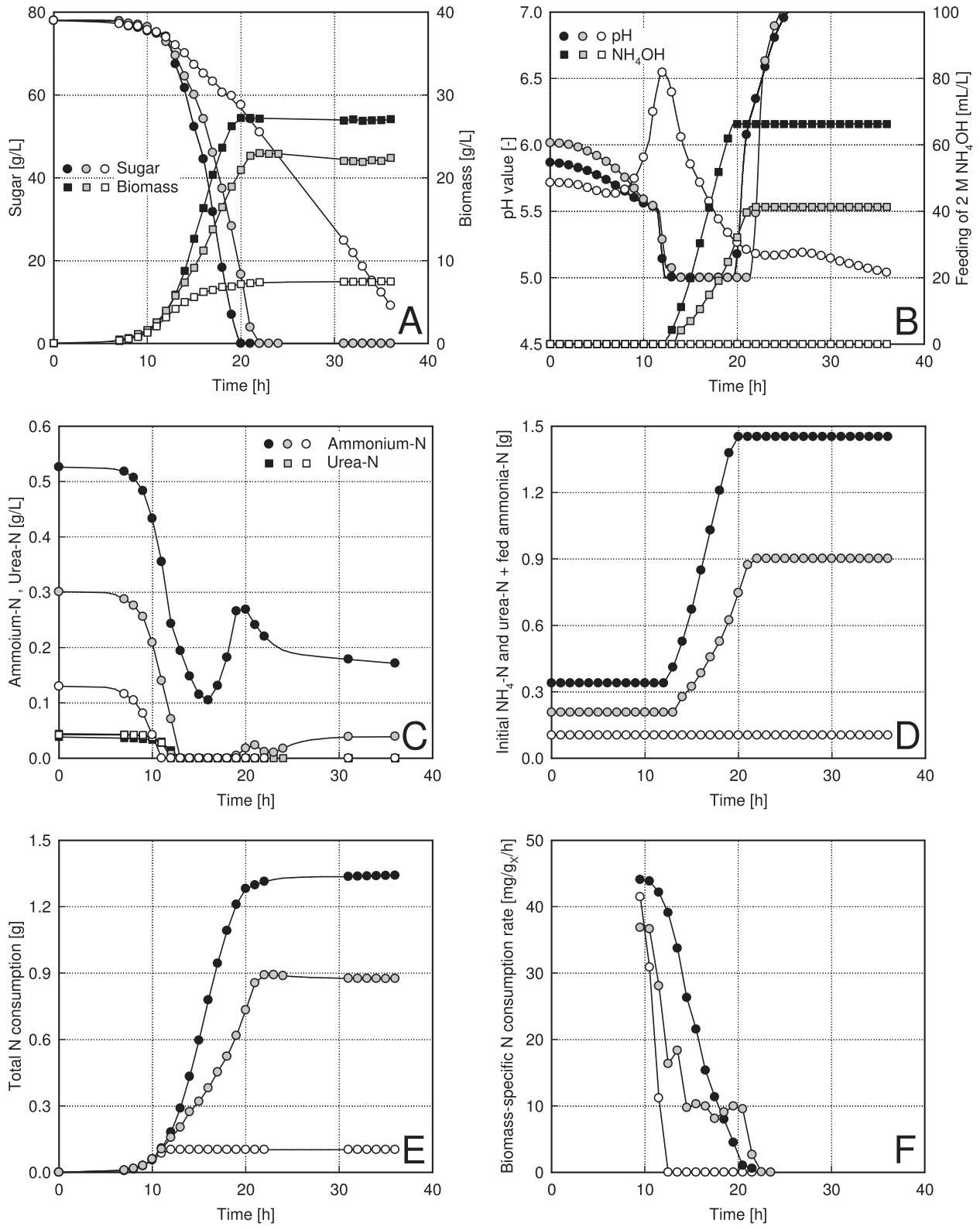

Figure 4 (See legend on next page.)

(See figure on previous page.)
Figure 4 Aerobic batch cultivation of *K. marxianus* DSM 5422 in stirred bioreactors using $(NH_4)_2SO_4$ and NH_4OH. Aerobic batch cultivation of *K. marxianus* DSM 5422 in a stirred bioreactor using various amounts of $(NH_4)_2SO_4$ and NH_4OH as sources of nitrogen. DW medium with 2 mL/L trace-element solution was supplemented with $(NH_4)_2SO_4$ or Na_2SO_4 (white symbols = 0.4 g/L Na_2SO_4; grey symbols = 1 g/L $(NH_4)_2SO_4$; black symbols = 2 g/L $(NH_4)_2SO_4$); the cultivation occurred in an 1-L stirred reactor at 1,200 rpm, 32°C, and aeration with 50 L/h; the pH was controlled to ≥5 with 2 M NH_4OH; the given growth rates were derived from measured CO_2 data.

$(NH_4)_2SO_4$ which was added as a pure substance to the autoclaved DW medium (0, 0.6, or 1.2 g). These processes were limited neither by oxygen (pO_2 always >10%) nor by sulfur (proven by residual sulfate).

In DW medium without an $(NH_4)_2SO_4$ supplement (Figure 4; white symbols), *K. marxianus* DSM 5422 grew on whey-borne nitrogen (0.13 g/L NH_4-N and 0.04 g/L urea-N), but this nitrogen was quickly depleted (Figure 4C) and the growth became N limited (Figure 4A). The pH temporally rose (Figure 4B) which seemingly correlated with urea consumption (Figure 4C). After depletion of all bioavailable N, the pH decreased only slowly due to a low metabolic activity (look at the sugar concentration in Figure 4A). Later, the pH stagnated above pH 5 and ammonia was thus not dosed (Figure 4B). The low availability of nitrogen (Figure 4D) caused restricted yeast growth (Figure 4A). The total N consumption and the N consumption rate were accordingly low (Figure 4E,F).

A supplement of 1 g/L $(NH_4)_2SO_4$ in the second experiment (Figure 4; grey symbols) increased the bioavailable N (Figure 4D) and allowed better yeast growth due to the higher initial NH_4-N which let the pH quickly decrease (Figure 4B). After depletion of this nitrogen, the pH stagnated at 5.05 for a while and then dropped below 5 where the pH controller started dosage of ammonia (Figure 4B). The added ammonia was assimilated immediately, and no ammonium accumulated in the medium (Figure 4C). That is, the yeast growth continued but at N-limited conditions as becoming visible from the low rates of ammonia dosage and N consumption (Figure 4D,F). This deficit of nitrogen slowed down growth and diminished the formed biomass (Figure 4A, Table 4). In the stationary period, some ammonium was released into the medium (Figure 4C) which was also observed by Ghaly and Kamal [26] at the cultivation of *K. marxianus* in whey and interpreted as decomposition of yeast biomass with release of NH_4-N into the medium.

A supplement of 2 g/L $(NH_4)_2SO_4$ in the third experiment (Figure 4; black symbols) increased the bioavailable N most (Figure 4D) and resulted in fast yeast growth and quick acidification. The feed of ammonia at pH <5 started before the initially added NH_4-N had been depleted (Figure 4C). The early start of ammonia dosage prevented limitation of yeast growth by nitrogen (NH_4-N always >100 mg/L), and the supply and uptake of nitrogen were well balanced (Figure 4C). The high rate of ammonia dosage corresponded with an accordingly fast growth and intensive N consumption (Figure 4D,E). The rate of N consumption became gradually smaller which is explained by the gently declining growth rate (possible reasons for this fading growth were discussed above). Urea was assimilated co-metabolically with the ammonium (Figure 4C).

The process with a supplement of 2 g/L $(NH_4)_2SO_4$ and ammonia dosage ran very similar to the process with 10 g/L $(NH_4)_2SO_4$; the final cell concentrations, formed biomasses, and the overall $Y_{X/S}$ values were nearly identical in both processes (Table 4) which demonstrates effective cultivation of *K. marxianus* with a pH-controlled feed of ammonia. Supplementing the medium with some $(NH_4)_2SO_4$ was however required for a quick acidification and for initiation of ammonia dosage. The amount of added $(NH_4)_2SO_4$ could be reduced by changing the set-point of the pH controller (e.g., to pH 5.5) so that dosage of ammonia starts earlier and avoids N-limited conditions even at a reduced $(NH_4)_2SO_4$ supplement, but some $(NH_4)_2SO_4$ is needed to cover the requirement for sulfur.

Nitrogen in biomass

The content of nitrogen in biomass grown at cultivation in the stirred bioreactor (Figures 3 and 4) is the inverse of the overall biomass yield for nitrogen: $x_N = 1/Y_{X/N}$. The overall $Y_{X/N}$ values were calculated from the produced biomass and the consumed nitrogen, assuming that only ammonium and urea were assimilated (Table 4). This calculation ignores that *K. marxianus* possibly hydrolyzes some whey-borne proteins and assimilates thus-formed peptides and amino acids. The N content of biomass depended on the extent of N limitation (Table 4): cultivation with enough nitrogen (process with 5 g/L urea and process with 2 g/L $(NH_4)_2SO_4$ plus dosed ammonia) gave x_N values of 81 and 83 mg/g, a slight deficit of nitrogen during the late growth stage (process with 10 g/L $(NH_4)_2SO_4$) resulted in x_N = 76 mg/g, distinct N limitation (process with 1 g/L $(NH_4)_2SO_4$ plus ammonia) yielded x_N = 67 mg/g, while severe N limitation (process without any N supplement) produced an x_N value of only 24 mg/g. A diminished N content of *K. marxianus* was also observed at limitation of growth by trace elements [10,11]. A decreased N content can be explained by a lowered portion of active biomass owing to intracellular storage of polysaccharides (details in [10,11]).

The N content can also be derived from the elemental composition of biomass. Several authors measured the cell composition for *K. marxianus* by elemental analyzers and transformed these data into biomass

formulae: $CH_{1.78}O_{0.75}N_{0.16}$ [45], $CH_{1.776}O_{0.575}N_{0.159}$ [53], $CH_{1.63}O_{0.54}N_{0.16}$ [54], $CH_{1.94}O_{0.76}N_{0.17}$ [55]. These formulae represent an N content of 88, 80, 91, or 83 mg/g. The fluctuations originate from measuring errors and from a variable cell composition depending on growth conditions [68].

Stoichiometry of yeast growth

Stoichiometric equations for describing the growth of *K. marxianus* has been derived here by using the method of Hensing et al. [39] and Mazutti et al. [69]. Such balancing requires a sum formula for biomass. The above-given formulae for *K. marxianus* biomass are restricted to C, H, O, and N as the predominating elements (derived from elemental analyses [45,53-55]). Here, the elements P, S, and K are included for more precision. The C, H, and O content was taken from the above-given biomass formulae (as averages), the N content of 82 mg/g was taken from own measurements at non-limited yeast growth, and the P, S, and K content was assumed with 10, 4, and 2 mg/g (own assimilation measurements). Combination of these data gives $CH_{1.78}O_{0.66}N_{0.158}P_{0.009}S_{0.0035}K_{0.0015}$ (yielding a molar mass of 27.027 g/mol).

Individual stoichiometric balance equations were derived for ammonium, urea, or ammonia as an N source, assuming respiratory growth (without formation of ethanol or ethyl acetate) of *K. marxianus* DSM 5422 with lactose as a substrate. The included stoichiometry coefficients were determined by balancing each element: seven balance equations were obtained containing nine unknown stoichiometric coefficients. This uncertain algebraic system was dissolved following Hensing et al. [39] by adding a proton balance and introducing the yield coefficient ($Y_{X/S}$ informs about the relation of assimilatory to dissimilatory substrate utilization and allows to establish the mass ratio between formed biomass and consumed lactose). $Y_{X/S}$ = 0.36 g/g was used here uniformly for all balances as found at non-limited growth with ammonium or ammonia (Table 4). Phosphate and sulfate were consumed in form of HPO_4^{2-} and SO_4^{2-} at the prevailing pH. Three equations were obtained for ammonium, ammonium hydroxide, or urea as an N source:

Protons are only formed during growth with ammonium which explains the observed substantial consumption of KOH by the pH controller with ammonium sulfate; the proton release equates to 0.28 g consumed KOH per g grown biomass and hence somewhat deviates from the measured KOH consumption (0.34 g_{KOH}/g_X). With NH_4OH or urea (Equations 4 and 5), the balances predict a slight alkalinization since OH^- ions are formed. The consumption of some KOH with urea as an N source is contradictory to this finding, but it should be kept in mind that the final pH was higher than the initial pH (Figure 3B); synthesis of organic acids (acetate, pyruvate, 2-oxoglutarate, and succinate were by-products of aerobic sugar metabolism of *K. marxianus* [16,68,70,71]) presumably caused KOH consumption, and consumption of these acidic metabolites after depletion of sugar alkalinized the medium. But such temporary metabolite accumulation was not considered at balancing. Another interfering effect originates from whey-borne lactate (ca. 4 g/L in DW medium [10]) whose microbial utilization also causes some alkalinization.

The balance equations allow to compare calculated with measured masses of consumed oxygen and formed carbon dioxide. The expected masses were calculated from the masses of utilized sugar. The measured masses (Table 4) were 1% to 20% smaller than predicted for unknown reason, but the ratio between formed CO_2 and consumed oxygen (the average RQ values) was ca. 1.02 mol/mol (Table 4) and agreed well with the predicted values (1.01 to 1.03 mol/mol).

Conclusions

Whey is poor in nitrogen and requires supplementation with an N source for effective production of yeast biomass. Ammonium sulfate, as usually applied for this reason, causes medium acidification by residual sulfate which requires pH control by alkaline substances to avoid growth inhibition. Application of ammonium carbonate instead of ammonium sulfate is not helpful since added $(NH_4)_2CO_3$ elevates the pH to inhibitory levels. *K. marxianus* DSM 5422 assimilates urea as an

$$C_{12}H_{22}O_{11} + 0.0410\ HPO_4^{2-} + 0.0160\ SO_4^{2-} + 0.0068K^+ + 7.3790\ O_2 + 0.7205\ NH_4^+ \rightarrow 4.5600\ CH_{1.78}O_{0.66}N_{0.158}P_{0.009}S_{0.0035}K_{0.0015} + 7.4400\ CO_2 + 8.0963\ H_2O + 0.6133H^+ \quad (3)$$

$$C_{12}H_{22}O_{11} + 0.0410\ HPO_4^{2-} + 0.0160\ SO_4^{2-} + 0.0068K^+ + 7.3790\ O_2 + 0.7205\ NH_4OH \rightarrow 4.5600\ CH_{1.78}O_{0.66}N_{0.158}P_{0.009}S_{0.0035}K_{0.0015} + 7.4400\ CO_2 + 8.7095\ H_2O + 0.1072\ OH^- \quad (4)$$

$$C_{12}H_{22}O_{11} + 0.0410\ HPO_4^{2-} + 0.0160\ SO_4^{2-} + 0.0068K^+ + 7.9189\ O_2 + 0.7205\ NH_2CONH_2 \rightarrow 4.5600\ CH_{1.78}O_{0.66}N_{0.158}P_{0.009}S_{0.0035}K_{0.0015} + 8.1600\ CO_2 + 8.3495\ H_2O + 0.1072\ OH^- \quad (5)$$

alternative N source. Consumption of urea means proton neutrality, medium acidification is minor, and only a little pH corrective is required. Moreover, the use of urea reduces the salt load (less inhibition, diminished environmental impact). Dosage of ammonia by the pH controller is a cost-saving alternative, but a suitable supplement of $(NH_4)_2SO_4$ is needed as a source of sulfur and for initiating dosage of ammonia.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CL and TU conceived of the study. EG, CL, and TU explored relevant literature. CL and TU designed the experiments. EG and TU conducted the experiments. CL performed data analysis. CL and TB drafted the manuscript. All authors read and approved the final manuscript.

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Dedication
This publication is dedicated to Prof. Dr. Andreas Zehnsdorf on the occasion of his 50th birthday.

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