Christian Löser
Christiane Haas
Wanqiong Liu
Sebastian Grah
Thomas Bley

Chair of Bioprocess Engineering,
Institute of Natural Materials
Technology, Technische
Universität Dresden, Dresden,
Germany

Research Article

Uptake of iron by *Kluyveromyces marxianus* DSM 5422 cultivated in a whey-based medium

The ability of *Kluyveromyces marxianus* for converting lactose into ethyl acetate offers a chance for the economical reuse of whey. Iron plays a significant role in this process as ester synthesis requires a low intracellular iron content, x_{Fe} . The iron content in turn is decreased by growth due to cell expansion and increased by iron uptake. Thus, the iron-uptake rate, ψ , is important for the considered process. Iron uptake by *K. marxianus* DSM 5422 was studied in aerobic cultivation on a whey-borne medium with varied initial iron content, in part combined with a feed of iron under intensive growth conditions. A possible precipitation of iron that would pretend iron uptake was verified not to have occurred. Regularly measured dissolved iron concentrations, $C_{Fe,L}$, allowed the x_{Fe} and ψ parameters to be obtained by model-based iron balancing. The achieved data were used for establishing a $\psi(C_{Fe,L}, x_{Fe})$ model. Mathematical simulations based on this iron-uptake model reproduced the performed cultivation processes. The created iron-uptake model allows for a future predictive system to be developed for the optimization of biotechnological ester production.

Keywords: Ethyl acetate / Iron adsorption / Iron uptake / *Kluyveromyces marxianus* / Modeling

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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1 Introduction

Whey arising in huge amounts during milk processing has turned from a waste to a valuable renewable resource in the field of White Biotechnology [1–3]. One possible application of whey is the microbial conversion of whey-borne lactose into ethyl acetate that is extensively used as an environmentally friendly solvent [4]. The yeasts *Wickerhamomyces anomalus*, *Candida utilis*, and *Kluyveromyces marxianus* are known for their ability to synthesize ethyl acetate from sugars or ethanol, but only the latter is able to consume lactose [5]. Some more properties predestinate *K. marxianus* for biotechnological applications, that is its “generally recognized as safe” status as a dairy yeast, its Crabtree-negative characteristics [5–7], its quick growth at a wide spec-

trum of sugars [8–10], and its distinct thermal tolerance enabling growth at elevated temperatures [8, 11]. The cultivation at high temperature promotes ester stripping, which again counteracts the accumulation of ethyl acetate up to toxic levels and allows for its process-integrated recovery [12]. Diverse strains of *K. marxianus* produce significant amounts of ethyl acetate [13]. The production of ethyl acetate by *K. marxianus* DSM 5422 has thoroughly been studied in many respects with special attention to the mechanism of ester synthesis in this strain [14–17].

The synthesis of ethyl acetate in *K. marxianus* and *C. utilis* is triggered by bioavailable iron: a deficit in iron limits yeast growth and initiates ester formation, while an excess in iron repressed ester synthesis [18–22]. The synthesis of ethyl acetate due to a lack of iron has also been confirmed for *K. marxianus* DSM 5422 [14, 15, 17]. Ester synthesis starts when the intracellular iron content falls below 30 μg iron per gram of dry biomass (estimated from the data published in [12, 15]). A low intracellular level of iron in *K. marxianus* reduces the metabolic flux of pyruvate into the citrate cycle due to both a reduced aconitase and succinate dehydrogenase activity (as both enzymes depend on iron) and a diminished nicotinamide adenine dinucleotide (NADH) reoxidation in the electron transport chain [16]. The accumulating pyruvate is diverted to ethyl acetate via two possible ways: (1) oxidative decarboxylation to acetyl-CoA, which

Correspondence: Dr. Christian Löser (christian.loeser@tu-dresden.de), Chair of Bioprocess Engineering, Institute of Natural Materials Technology, Technische Universität Dresden, 01062 Dresden, Germany

Abbreviations: AAS, atomic absorption spectrometry; NADH, nicotinamide adenine dinucleotide

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is then condensed with ethanol by alcohol acetyltransferase, and (2) reductive decarboxylation to acetaldehyde, which is spontaneously condensed with ethanol to a hemiacetal being thereafter oxidized by alcohol dehydrogenase [23–27]. The formation of ethyl acetate via the reverse esterase reaction from acetate and ethanol, as repeatedly postulated in the literature (see, e.g., [5, 27]), is unlikely as the hydrolysis of ethyl acetate is thermodynamically favored over its synthesis [26].

The intracellular iron content, x_{Fe} , is influenced by two processes: iron uptake and yeast growth. Iron uptake from the surrounding culture medium increases x_{Fe} , while growth expands the cell volume and diminishes x_{Fe} . Then again, the intracellular iron content controls yeast growth as iron is a mineral substrate being stored in the cell (such as nitrogen and phosphorous in microalgae [28]). Stored iron ensures continued growth even after the depletion of iron in the culture medium [15]. In general, the uptake of iron by the cells depends on two factors: (1) the bioavailability of iron, and (2) the charge status of the iron storage. Iron is only taken up when dissolved iron is available and the iron pools are not yet completely filled. The iron uptake itself changes the dissolved iron concentration in the medium. These facts demonstrate the complex interrelation between extracellular iron, intracellular iron, specific growth rate, and the iron-uptake rate.

The uptake of iron by yeasts was almost exclusively studied for *Saccharomyces cerevisiae* and mainly focused on the mechanism of iron uptake rather than the kinetics [29–34]: *S. cerevisiae* exhibits membrane-bound reductases for transforming media-borne Fe^{3+} ions (the predominating iron ion under aerobic conditions) into Fe^{2+} ions that are taken up either by the low-affine Fet4 transporter, acting at high iron concentrations, or by a high-affine system (Fet3 oxidase combined with the Ftr1 transmembrane permease) being active at low iron concentrations. *S. cerevisiae* also acquires Fe^{2+} via the nonspecific transition metal transporters. *S. cerevisiae* does not synthesize siderophores but uses such compounds produced by other organisms for iron absorption [35]. The iron-uptake rates for *S. cerevisiae*, for the most part measured in short-term experiments under nongrowth conditions, were given related to the cell numbers but not the biomass (e.g. [32, 36–38]).

Here, we report for the first time on iron uptake by *K. marxianus* DSM 5422 at aerobic batch cultivation in a stirred bioreactor using whey-borne medium of various initial iron concentrations and, in part, at a feed of iron in the linear growth period. The scientific progress consists of studying the iron uptake in real long-term cultivation processes but not in short-term experiments under nongrowth conditions, as is the norm. The time-dependent iron content of biomass and the iron-uptake rate were calculated from repeatedly analyzed liquid-phase iron concentrations. Our findings might improve the understanding of the interrelation between iron uptake, the extracellular iron content, and the intracellular iron level during yeast growth. The observations are formulated in a model that finally enables the establishment of a predictive system for the optimization of the biotechnological production of ethyl acetate.

2 Modeling

Here, modeling focuses on two subjects: (1) the illustration of the complex interaction between the specific growth rate, the iron-uptake rate, the dissolved iron concentration, and the iron content of yeasts, and (2) the derivation of equations for the evaluation of the measured iron data. The presented model applies to the following conditions: batch mode cultivation, homogeneously mixed suspension, restriction of growth by sugar, aerobic conditions, and repression of ester synthesis by higher intracellular iron levels. This model considers a temporal feed of iron, takes changes of the suspension volume into account, and differentiates between liquid-phase and suspension volume.

The following equations describe the temporal changes in biomass, sugar, and iron concentration during the cultivation process (derived in Supporting information 1):

$$\frac{dC_X}{dt} = \mu \cdot C_X - C_X \cdot \frac{F_{pH} + F_{Fe} - F_{Ev.}}{V} \quad (1)$$

$$\frac{dC_S}{dt} = -\frac{\mu \cdot C_X}{Y_{X/S}} - C_S \cdot \frac{F_{pH} + F_{Fe} - F_{Ev.}}{V} \quad (2)$$

$$\frac{dC_{Fe}}{dt} = -\psi \cdot C_X - C_{Fe} \cdot \frac{F_{pH} + F_{Fe} - F_{Ev.}}{V} + C_{Fe,L,0} \cdot \frac{F_{Fe}}{V} \quad (3)$$

These concentrations denote grams of the respective compound related to the suspension volume. In the case of sugar and iron, these variables are only fictive concentrations, imaging that both compounds are homogeneously distributed in the suspension, although they are actually only dissolved in the aqueous phase. The temporal changes of both concentrations are determined by yeast growth ($\mu \cdot C_X$), iron uptake ($\psi \cdot C_X$), dilution and concentration effects (the second right-hand term), and by iron feed (the third term in Eq. (3)). The suspension volume increases by the dosage of both a pH corrective solution and an iron stock solution but decreases by evaporation of water and sampling:

$$\frac{dV}{dt} = F_{pH} + F_{Fe} - F_{Ev.} - F_{Sampling} \quad (4)$$

The feed of KOH regulated by the pH controller (F_{pH}) was recorded during the experiment, whereas the iron stock solution was only fed in some tests ($F_{Fe} = 1.21$ mL/h). Losses of water by evaporation depend on aeration (here, $F_{Ev.} = 0.4$ mL/h). Sampling occurred discretely but is formulated as a continuous process during the sampling periods ($F_{Sampling} = 2.4$ mL/h).

The suspension volume (V) is composed of the aqueous-phase volume (V_L) and the yeast cell volume (V_{Xs}). Sugar and soluble iron are only present in the aqueous phase, and the measured concentrations can distinctly deviate from the suspension concentrations. The higher the cell concentration is, the larger this deviation becomes. The conversion of the measured liquid-phase concentrations to the respective suspension concentrations is based on the following equations:

$$C_S = C_{S,L} \cdot (1 - \varphi_{Xs}) \quad (5)$$

$$C_{Fe} = C_{Fe,L} \cdot (1 - \varphi_{Xs}) \quad (6)$$

Parameter φ_{X^*} , the volume fraction of the suspended biomass, correlates with the cell concentration and depends on both the density (ρ_{X^*}) and dry mass/wet mass ratio (Y_{X/X^*}) of intact cells:

$$\varphi_{X^*} = \frac{C_X}{\rho_{X^*} \cdot Y_{X/X^*}} \quad (7)$$

Extracellular and intracellular iron species are often not equilibrated for two reasons: (1) luxury iron uptake and intracellular storage, and (2) rate-limited transport of iron into the cells. Iron uptake and yeast growth based on intracellular iron are considered here as decoupled processes. Essential trace metals are taken up quickly at the onset of cultivation and stored in the cell till their depletion in the culture medium, so that further growth is based on the stored metal being accompanied by a steady reduction of the intracellular metal content. Such a process has already been described for zinc during the fermentation of *Saccharomyces* strains in malt wort [39,40], and should also be valid for iron.

A differentiation of iron into extracellular and intracellular iron is formulated using the dual-compartment model. This model with the cell volume and the medium volume as the two compartments has originally been developed for describing algal growth in dependence on phosphorus [41]; P is taken up by the microalgae and stored in the cell, and then the deposited P is used for growth. This model is combined with the cell quota model developed for storable nutrients, such as N, P, and vitamin B₁₂ [28], where cell quota means that the mass of a stored nutrient is related to the biomass dry weight. Hence, the following equation is obtained:

$$\frac{dx_{Fe}}{dt} = \psi - \mu \cdot x_{Fe} \quad (8)$$

The temporal change in the iron content of yeasts (x_{Fe} , given in μg iron per g dry biomass) depends on the iron-uptake rate (ψ) and the dilution of the intracellular iron during growth ($\mu \cdot x_{Fe}$). The iron uptake increases x_{Fe} and reduces the dissolved iron concentration as shown in Eq. (3). The ψ and μ variables do not only influence several processes but also depend on other variables (see the details below).

3 Materials and methods

3.1 Microorganism and culture media

Kluyveromyces marxianus DSM 5422 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). This yeast strain proved to be a potent producer of ethyl acetate and was repeatedly used for studying various aspects of ester synthesis from whey-borne lactose [5,11–17,42]. The inocula were obtained by yeast cultivation on YGC agar (Roth GmbH, Germany) for 2 days at 32°C.

The medium was based on partially demineralized whey permeate processed by the Sachsenmilch AG (Leppersdorf near Dresden, Germany) as described in [17]. The whey was diluted

by the same volume of deionized water and the mixture supplemented with 10 g/L (NH₄)₂SO₄. The medium was heat sterilized in a 1-L Schott bottle, and the clear upper phase was transferred to another bottle and termed “DW basic medium” [17]. Dissolved inorganic ions were analyzed earlier [17,42].

3.2 Bioreactor cultivation

Cultivation occurred in a 1-L stirred bioreactor made of glass and controlled by an ADI 1030 Biocontroller (Applikon), as described previously [42]. The reactor was charged with 1.2 mL Antifoam A (Fluka), autoclaved for 20 min at 121°C, its interior dried by a sterile air flow, and filled with 600 mL sterile DW basic medium. Then, 1.2 mL sterile trace element solution was added being composed like a normative trace element solution [17] but with varied iron content. The reactor was operated at 32°C, 1200 rpm, and an air flow of 50 L/h (at 0°C and 101 325 Pa, water content 0.005 L/L). The medium was inoculated using approximately 0.006 g biomass according to [17]. The pH was controlled to ≥ 5 by 2 M KOH. Samples of 2.4 mL were repeatedly withdrawn; 0.4 mL were used for *OD*_{600nm} measurements and 2.0 mL were processed according to [42] for determining the biomass dry weight and concentrations of dissolved compounds. Final sampling of 4 × 40 mL suspension enabled a precise biomass analysis.

In four bioreactor experiments, a heat-sterilized iron stock solution with 327.6 mg/L iron was applied (FeSO₄·7H₂O dissolved in 0.03 N HCl). After 14 h of regular cultivation, the stock solution was supplied for 7 h with 1.21 mL/h using an LA-120 syringe pump (Landgraf Laborsysteme, Hannover). The exact amount of supplied stock solution was obtained by weighing the syringe before and after the feeding period.

3.3 Data evaluation

The data measured in bioreactor experiments were analyzed regarding the following parameters: exhaust gas flow, O₂ consumption, CO₂ formation, and RQ value according to Duboc and von Stockar [43]. The specific growth rate (μ) was calculated for $t \dots t + \Delta t$ intervals in four different ways: from *OD*_{600nm} values, from biomass data, and from the moles of cumulatively consumed O₂ and formed CO₂: $\mu = \ln[OD_{600nm}(t + \Delta t)/OD_{600nm}(t)]/\Delta t$, $\mu = \ln[C_X(t + \Delta t)/C_X(t)]/\Delta t$, $\mu = \ln[n_{O_2}(t + \Delta t)/n_{O_2}(t)]/\Delta t$, and $\mu = \ln[n_{CO_2}(t + \Delta t)/n_{CO_2}(t)]/\Delta t$. The latter two took losses of biomass at sampling into account.

The intracellular iron content, x_{Fe} , was calculated by balancing the mass of iron based on measured *C*_{Fe,L} data. This balancing starts with the definition of x_{Fe} :

$$x_{Fe}(t) = \frac{m_{Fe}^{int}(t)}{m_X(t)} \quad (9)$$

The mass of internal iron is the total mass of iron at the beginning plus the mass of supplied iron minus the masses of extracellular iron and withdrawn iron at sampling:

$$x_{Fe}(t) = \frac{m_{Fe}^{total}(t=0) + \int_0^t \dot{m}_{Fe}(Supply) dt - m_{Fe}^{ext}(t) - \sum_0^t m_{Fe}^{total}(Sampling)}{m_X(t)} \quad (10)$$

Integration and substitution of masses by concentrations yields:

$$x_{Fe}(t) = \frac{m_{Fe}^{total}(t=0) + C_{Fe,L,0} \cdot F_{Fe} \cdot \Delta t_{Feed} - C_{Fe}(t) \cdot V(t) - \sum_0^t m_{Fe}^{total}(Sampling)}{C_X(t) \cdot V(t)} \quad (11)$$

Herein, Δt_{Feed} is the time interval of iron feed till time t (only in some experiments, and only in a given period). The mass of initially present iron (dissolved and in the inoculum) is:

$$m_{Fe}^{total}(t=0) = C_{Fe}(t=0) \cdot V(t=0) + C_X(t=0) \cdot x_{Fe}(t=0) \cdot V(t=0) \quad (12)$$

The iron in the inoculum is negligible, since $C_X(t=0)$ was small. The loss of both extracellular and intracellular iron by sampling until time t is:

$$\sum_0^t m_{Fe}^{total}(Sampling) = \sum_{i=1}^{j-1} (C_{Fe}(t_i) \cdot V_{Sample} + C_X(t_i) \cdot x_{Fe}(t_i) \cdot V_{Sample}) \quad (13)$$

The $C_X(t)$ data were measured, the $C_{Fe}(t)$ values were calculated from the measured $C_{Fe,L}(t)$ data using Eq. (6), and the $V(t)$ values were calculated using Eq. (4). The specific iron-uptake rates were received from Eq. (8) by converting the dx_{Fe}/dt expression to a difference quotient (the variables marked with $(t \dots t + \Delta t)$ represent the averages for this time interval):

$$\psi(t \dots t + \Delta t) = \frac{x_{Fe}(t + \Delta t) - x_{Fe}(t)}{\Delta t} + \mu(t \dots t + \Delta t) \cdot x_{Fe}(t \dots t + \Delta t) \quad (14)$$

All confidence intervals of data were calculated for a confidence level of 95%.

3.4 Dry mass fraction and density of intact biomass

The ρ_{X*} and $Y_{X/X*}$ parameters were analyzed using biomass freed from adhesive water. Intact *K. marxianus* DSM 5422 biomass from bioreactor cultivations was separated from portions of 40 mL suspension by a Biofuge Stratos centrifuge (3 min at 15 000 rpm; Heraeus, Hanau) and then washed with 40 mL PBS. The pellet was resuspended in 40 mL deionized water and the

suspension was subjected to vacuum filtration for 4 min (filtration unit with 16612 type pump; regenerated cellulose filters of Ø 50 mm and 0.45 µm pore size; Sartorius Stedim Biotech, Göttingen). The filter was washed using 5 mL deionized water at a continued filtration for 2 min, and adhesive water was removed by filtration for an additional 7 min (procedure optimized in previous tests).

A defined amount of so-treated biomass (ca. 4 g) was put on aluminum foil, dried at 105°C, and the residual dry mass weighed. The $Y_{X/X*}$ parameter is the ratio between the dry weight and wet weight. The ρ_{X*} parameter was determined by using a pycnometer bottle of $V_{total} = 50$ mL. The pycnometer was balanced, filled with approximately 4 g biomass using a funnel and glass rod, and balanced again to determine the filled-in biomass (m_{X*}). The loaded pycnometer was filled up with water at 25°C and balanced again yielding the mass of biomass plus water (m_{total}). The density of biomass was calculated as follows: $\rho_{X*} = m_{X*}/V_{X*} = m_{X*}/(V_{total} - V_{Water}) = m_{X*}/(V_{total} - (m_{total} - m_{X*})/\rho_{Water})$.

3.5 Extraction of iron adsorbed to biomass

This method detects iron that is fixed to the yeast cells adsorptively or as particulate matter. The published iron extraction methods were critically reviewed and several procedures tested, yielding the following optimized method. A total of 40 mL cell suspension was centrifuged (Heraeus Biofuge Stratos, 3 min, 15 000 rpm) and the pellet was washed twice with 40 mL PBS (the last supernatant was stored at −20°C). The washed pellet was filled with PBS to a volume of 16 mL and the mixture was homogenized. Three 15-mL centrifuge tubes were each filled with 4 mL of this suspension. Then, 5 mL 100 mM sodium dithionite in water and 1 mL of a complexing agents mixture (containing 100 mM EDTA and 100 mM citrate, pH adjusted to 5 by 2 M NaOH) were mixed with the suspension, and the mixture was left to react for 10 min at 25°C. The suspensions were centrifuged as above and the supernatants were stored at −20°C. Another three batches were prepared for detecting iron in the extractant with water instead of cell suspension. The iron content of all liquids was analyzed by atomic absorption spectrometry (AAS). The amount of iron in real extracts was reduced by the iron in adhering wash water and in the extractant, and then related to

the dry mass of extracted biomass, yielding microgram adhered iron per gram of dry biomass.

3.6 Analyses

Biomass dry weight was determined by separating yeasts from suspensions via centrifugation, washing the pellet two times with water, and drying at 105°C. Sugar was determined by the DNS method [44] with lactose as a standard. Volatile organic compounds were analyzed by GC [42]. The OD_{600nm} was measured using a DU 520 photometer (Beckman).

The iron content of aqueous samples was analyzed by AAS according to the DIN 38406–32 method [45] using a Varian AA 240 spectrometer adjusted to the following parameters: wave length 248.3 nm, slit width 0.2 nm, sample flow 4.5 mL/min, preflush 5 s, signal detection 3×5 s. The sample injection system was flushed with water after each analysis until the yellow flame color had faded. The samples were routinely diluted using 30 mM HNO_3 (Fe content of the acid ca. 0.5 $\mu g/L$) in a 1:3 volume ratio for lowering the salt load. Calibration occurred using a commercial iron solution (Roth GmbH), diluted with 30 mM HNO_3 to iron contents of 0.05–6 mg/L iron. Calibration happened at the beginning and after every 25–30 samples. Zero-point drift was handled by blank analysis (30 mM HNO_3) of every three samples. Data processing was done in an offline mode, using the following function: $C_{Fe,L} = a \cdot \Delta A_{248,3nm} + b \cdot (\Delta A_{248,3nm})^2$ (a and b were recalculated for each measuring session). Four-fold analysis of each sample resulted in 12 separate $\Delta A_{248,3nm}$ values (with an inaccuracy of approximately 3% at a 95% confidence level).

4 Results

4.1 Dry mass fraction and density of biomass

The dry mass fraction and density of intact *K. marxianus* DSM 5422 biomass are used for calculating the volume fraction of biomass, φ_{X*} , in cell suspensions (see Eq. (7)). The φ_{X*} parameter is required for converting the measured aqueous-phase-related concentrations into suspension-volume-related concentrations (Eq. (6)). The latter are again needed for balancing the iron (Section 3.3). The determination of $Y_{X/X*}$ and ρ_{X*} was based on intact yeast biomass, freed from adhesive water by vacuum filtration.

The $Y_{X/X*}$ parameter was measured in seven replicate tests using biomass from two bioreactor cultivations done in DW basic medium with surplus iron. Both biomasses delivered identical results and were combined to one average value: $Y_{X/X*} = 0.2261 \pm 0.0017$ g/g. The dry-mass fraction is lower than a quarter of the total biomass. This result let us speculate about an incomplete removal of adhesive water, but extended vacuum filtration did not change the value. The water content of the intact yeast cells equates to $1 - Y_{X/X*}$, yielding 0.7739 g/g.

The density of *K. marxianus* DSM 5422 biomass was determined in five replicates with yeasts from the same cultivations as used for $Y_{X/X*}$ determination. Both biomasses gave identical values and were therefore combined to one average: $\rho_{X*} = 1066.6 \pm$

1.1 g/L. This value is somewhat higher than the density of water and meets the expectations.

Cultivation of *K. marxianus* DSM 5422 in DW basic medium with surplus iron yields a final biomass concentration of approximately 25 g/L (Table 1), which corresponds to a volume fraction of 0.104 L/L (according to Eq. (7)). This means that more than 10% of the suspension volume is occupied by yeast cells. Consequently, the suspension-volume-related iron concentrations are up to 10% lower than the measured aqueous-phase concentrations (Eq. (6)). Ignoring this fact at balancing the iron would result in defective x_{Fe} and ψ values. This argues for the relevancy of this consideration.

4.2 Iron uptake by *K. marxianus* DSM 5422

Iron uptake by *K. marxianus* DSM 5422 was studied under aerobic cultivation conditions in a 1-L stirred bioreactor using DW basic medium with a varied initial iron content and, in part, with a feed of iron stock solution in the period of intensive growth. A typical cultivation process is shown in Fig. 1 for a medium with 5.325 mg/L iron. All other bioreactor processes run similarly.

The processes were always fully aerobic with a $pO_2 \geq 50\%$ air saturation. A significant formation of ethyl acetate was suppressed by an intracellular iron content of $x_{Fe} > 30 \mu g/g$ in all experiments. Growth was at first exponential but after about 14 h switched to a linear mode and nearly stopped after depletion of sugar (Fig. 1A). Further growth was based on the formed metabolites (ethanol, pyruvate, glycerol [14]). Oxygen consumption and CO_2 formation correlated with growth and partially resulted from maintenance (Fig. 1B). Acidification due to ammonium consumption for biomass synthesis decreased the pH [46], but controlled dosage of 2 M KOH assured a $pH \geq 5$ (with an overall KOH consumption of approximately 52 mL; Table 1). After depletion of sugar, acidification stopped and the pH gradually increased to a final value of pH 7.6.

The respiratory quotient, RQ , was calculated from the data depicted in Fig. 1B: $RQ = \Delta n_{CO_2} / \Delta n_{O_2}$. Sugar is at first utilized respiratorily ($RQ \approx 1.02$ mol/mol), but around 3 h prior to sugar depletion, metabolism became partially fermentative ($RQ \approx 1.15$ mol/mol) for unknown reasons corresponding to some ethanol synthesis (Table 1). After sugar depletion, yeasts switched to the utilization of excreted metabolites; the RQ value quickly dropped to approximately 0.8 mol/mol and then successively returned to 1 mol/mol. The average RQ value for the total process was 1.01 mol/mol (Table 1), since the increased RQ in the respiro-fermentative period is compensated by a lower RQ at metabolite consumption.

Changes of the suspension volume in the depicted experiment occurred by a feed of a pH corrective, evaporation of water, and sampling (Fig. 1C). The overall change of the suspension volume was low due to the compensational effect of KOH dosage and sampling (both amounted to approximately 50 mL in the depicted test), but a dilution by KOH dosage and a loss of material by sampling are of importance and have to be taken into account when balancing the iron uptake.

The specific growth rate was calculated in four different ways. The obtained $\mu(t)$ courses are similar and distinctly exhibit the switch from an exponential to a linear growth mode (Fig. 1D).

Table 1. Parameters of cell growth, sugar consumption, product formation, and iron uptake during the aerobic batch cultivation of *K. marxianus* DSM 5422 in a 1-L stirred bioreactor using 0.6 L DW basic medium supplemented with trace element solution resulting in different initial iron concentrations

Parameter	Cultivation process							
	A	B	C	D	E	F	G	H
Iron, supplied during the process [mg]	0.000	0.000	0.000	0.000	2.775	2.777	2.777	2.772
Iron, initially present, $C_{Fe,L}(t=0)$ [mg/L]	2.167	3.211	4.240	5.325	2.145	3.250	4.284	5.342
Iron, available in total [mg]	1.305	1.936	2.559	3.217	4.046	4.737	5.363	6.000
μ_{max} calculated from CO ₂ data [h ⁻¹]	0.534	0.534	0.561	0.551	0.544	0.544	0.546	0.528
μ_{max} calculated from O ₂ data [h ⁻¹]	0.538	0.531	0.548	0.545	0.534	0.534	0.541	0.524
Duration of exponential growth, t_{crit} [h]	13.25	13.98	13.37	13.87	14.02	13.47	13.50	14.05
Biomass at t_{crit} [g]	3.74	3.85	3.34	3.77	4.03	3.65	3.81	3.79
Moment of sugar depletion [h]	20.3	20.5	20.2	20.2	20.3	20.3	20.3	21.6
Consumed 2 M KOH [mL]	49.9	52.3	54.0	51.3	50.5	51.2	52.7	52.0
Maximum ethanol content in the medium [g/L]	0.93	0.79	1.04	0.79	0.87	0.89	1.04	0.86
Maximum ester content in the medium [g/L]	0.06	0.09	0.10	0.11	0.19	0.05	0.05	0.07
Consumed sugar [g]	42.9	41.7	44.1	42.7	41.2	43.0	43.6	43.7
Final biomass concentration [g/L]	25.7	24.9	25.2	26.0	25.4	25.1	25.1	24.3
Formed biomass [g]	15.7	15.3	15.7	15.9	15.7	15.7	15.6	15.1
Growth yield, $Y_{X/S}$ [g/g]	0.365	0.370	0.355	0.373	0.381	0.366	0.358	0.346
Formed carbon dioxide [mol]	0.685	0.673	0.675	0.688	0.680	0.678	0.697	0.672
Consumed oxygen [mol]	0.676	0.669	0.672	0.686	0.674	0.671	0.689	0.660
Average RQ value [mol/mol]	1.014	1.007	1.004	1.004	1.009	1.011	1.011	1.018
$C_{Fe,L}$ after sugar depletion [mg/L]	0.113	0.105	0.325	0.572	1.890	3.566	4.611	5.897
$C_{Fe,L}$ after 24 h of cultivation [mg/L]	0.168	0.126	0.292	0.375	1.663	3.245	4.331	5.846
$C_{Fe,L}$ after 36 h of cultivation [mg/L]	0.150	0.162	0.340	0.443	1.626	3.251	4.459	5.835

Note: Processes without (A–D) or with (E–H) a feed of iron stock solution are as explained in Fig. 2. Cultivation conditions are as stated in the legend of Fig. 1.

The content of dissolved iron in the aqueous phase is mainly changed by the iron uptake of yeasts as well as the feed of iron during the process in some experiments (Fig. 2A). However, temporal variation of the liquid volume also influences the dissolved iron concentration to some degree (Eqs. (3) and (4)). In addition, the growing yeasts occupy more and more of the suspension volume, so that the iron concentrations related to the suspension volume substantially deviate from the measured iron concentrations (up to 10.4%; compare Fig. 2A with Fig. 2B). Differentiation between $C_{Fe,L}$ and C_{Fe} is therefore of great importance for a precise iron balancing.

In experiments without an iron feed, yeasts could only uptake iron that was initially added to the medium. The higher the cell density became, the faster the iron concentration dropped (Fig. 2). The lower the initial iron concentration was, the earlier the dissolved iron became nearly depleted. Despite a varying iron availability, the growth was not significantly influenced in single experiments, neither concerning the rate nor regarding the formed biomass (compare the moments of sugar depletion and the $Y_{X/S}$ parameters in Table 1). But the different availability of iron influenced the intracellular iron content of yeasts (Section 5.3).

The feed of iron during the process (from 14 to 21 h with a rate of approximately 0.4 mg/h) did not change the growth behavior (Table 1), but modified the $C_{Fe,L}(t)$ and $C_{Fe}(t)$ courses. At first, the iron concentration diminished in the same manner as in experiments without an iron feed. After starting the feed, the iron concentration increased rather than further decreased (iron feed > iron uptake). Later, at higher cell densities, the iron

uptake rose and slowed the increase in iron concentration or even slightly decreased it. Slowed-down growth (at approximately 18 h) resulted in a weakened iron uptake and a second rise of dissolved iron. After stopping the iron feed at 21 h, the iron content dropped again due to some continued iron uptake. In the period from 24 to 36 h, the dissolved iron did not change, although the pH rose to approximately 7.6.

4.3 Adsorption of iron to biomass

In this work, microbial iron uptake is derived from dissolved iron concentrations, repeatedly measured during yeast growth, by balancing the iron as shown in Section 3.3. This method requires reliable differentiation between extracellular and intracellular iron. Iron adsorbed to the cell surface and iron chemically precipitated could be misleadingly interpreted as iron taken up by microbes [47]. Such adsorbed and precipitated iron is efficiently identified by extraction and iron analysis of the extracts.

The extraction method employed here is based on several iron extraction methods that were formerly applied to marine phytoplankton [47–49] or *S. cerevisiae* [32, 36, 50, 51]. In these methods, complexing agents (EDTA, citrate), often combined with a reducing agent (TiCl₃, oxalate, dithionite), were used for iron mobilization. Several extraction procedures were tested with *K. marxianus* DSM 5422 biomass by varying the species and concentrations of reducing and complexing agents (not shown); extraction with 50 mM dithionite, 10 mM EDTA, and 10 mM

Figure 1. (A) Sugar and biomass concentration, (B) consumed oxygen and formed carbon dioxide, (C) volumes of withdrawn samples, supplied pH corrective, evaporated water and the suspension, and (D) specific growth rate calculated in various ways during the aerobic batch cultivation of *K. marxianus* DSM 5422 in a 1-L stirred bioreactor using 0.6 L DW basic medium supplemented with trace element solution resulting in an initial iron content of 5.325 mg/L; cultivation at 1200 rpm, 32°C, aeration with 50 L/h (standard conditions), pH ≥ 5. The measured dissolved iron concentrations are shown in Fig. 2A.

Figure 2. (A) Dissolved iron concentration as measured, $C_{Fe,L}$, and (B) dissolved iron concentration related to the suspension volume, $C_{Fe,S}$, during the aerobic batch cultivation of *K. marxianus* DSM 5422 in a 1-L stirred bioreactor using 0.6 L DW basic medium supplemented with trace element solution resulting in different initial iron concentrations. The individual symbols refer to the varied initial dissolved iron concentrations in the medium; processes without (white symbols) or with (gray symbols) a feed of iron stock solution ($F_{Fe} = 1.21$ mL/h from 14 to 21 h, labeled by the gray area); cultivation conditions as stated in the legend of Fig. 1.

citrate in the mixture was most effective. An inspection of so-treated yeasts by flow cytometry demonstrated that the extraction never affected the yeast cells; neither a loss of cells nor a formation of cell debris was detected (detailed data not shown). Intact *K. marxianus* DSM 5422 cells were spiked with freshly precipitated $FeO(OH)$, yielding 200 μ g iron per gram dry biomass, and then subjected to the optimized extraction method; the added particulate iron was completely extracted.

Iron precipitation could be significant in the operated experiments for several reasons: initially added ferrous iron ($C_{Fe,L}(t = 0) = 2.15\text{--}5.34$ mg/L) is quickly oxidized under aerobic conditions to form ferric iron, and the given pH conditions ($5.0 \leq pH \leq 7.6$) imply the risk of ferric iron precipitation. The highest risk for iron precipitation occurs at the highest pH at the end of cultivation. Biomass was therefore harvested at $t = 36$ h and was subjected to iron extraction. AAS analysis provided some iron in

the extracts, but most of this iron resulted from the used chemicals and some iron came from adhering wash water. These two iron fractions were subtracted and the residual iron was related to the dry mass of the extracted yeasts. The amount of extracted iron was low in all cultivations ranging from 0.1 to 1.2 $\mu\text{g/g}$, or was 0.7 $\mu\text{g/g}$ on an average. This demonstrates that extracellular adsorption and precipitation of iron was insignificant. The applied method of iron balancing has thus been proven as a valid concept.

5 Discussion

5.1 Growth behavior

Growth of *K. marxianus* DSM 5422 in whey-based media is influenced by many factors, such as medium composition [42], availability of sugar [42], oxygen [16], nitrogen [46], iron [14, 15, 17], zinc [17], copper [17], the temperature [11], and the inhibitory effect of ethyl acetate [12]. Here, only limitation of growth by sugar and an unknown factor will be considered by using the gamma concept [52]:

$$\mu = \mu_{\max} \cdot \gamma_{\mu}(C_{S,L}) \cdot \gamma_{\mu}(x_U) \quad (15)$$

The growth-limiting effect of iron is not considered, as iron was always sufficiently available in the here presented experiments. The limiting effect of sugar is described by a Monod expression with the half-saturation constant K_S :

$$\gamma_{\mu}(C_{S,L}) = \frac{C_{S,L}}{C_{S,L} + K_S} \quad (16)$$

The K_S values of yeasts for sugar are typically low. For example, the K_S values of *K. marxianus* for glucose were found to be 0.065 g/L [53] and 0.23 g/L [54]. In contrast, the published K_S values of *K. marxianus* for lactose extend over a wide range: 0.012 g/L [55], 1.2 g/L [56], 10.5 g/L [57], 16 g/L [58], 20 g/L [59]; most of these K_S values are implausibly high, which could be explained by the unsuitable mode of determination (the adaptation of the model to the measured data via multi-parameter variation). Here, the $\gamma_{\mu}(C_{S,L})$ term does not describe the precise μ – $C_{S,L}$ relation, but acts as a trigger from full growth with sugar to no growth after sugar depletion. The K_S value of *K. marxianus* DSM 5422 for lactose is definitely small, since the measured $C_{S,L}(t)$ curves exhibit a sharply bending course shortly before sugar depletion (Fig. 1A, [17, 46]), and is estimated to be $K_S \approx 0.1$ g/L. The measured O_2 consumption and CO_2 formation curves confirm this view (see Fig. 1B).

Growth of *K. marxianus* DSM 5422 in DW basic medium starts exponentially but later continues in a linear mode (Fig. 1A, [17, 46]). Sugar, oxygen, nitrogen, phosphorous, sulfur, potassium, iron, and other trace elements are ruled out as a growth-limiting factor since these compounds were present in excess. Limitation of growth by a yet unknown compound was therefore postulated, which is taken into account in Eq. (15) by the $\gamma_{\mu}(x_U)$ function, where x_U stands for the intracellular content of the unknown storable factor. The $\gamma_{\mu}(x_U)$ function amounts to 1 as long as growth is not limited by compound U (exponential growth); however, $\gamma_{\mu}(x_U)$ decreases when the mass

of grown yeasts, m_X , exceeds the critical value, $m_{X,\text{crit}}$ (linear growth):

$$\gamma_{\mu}(x_U) = \begin{cases} 1, & m_X < m_{X,\text{crit}} \\ \frac{m_{X,\text{crit}}}{m_X}, & m_X \geq m_{X,\text{crit}} \end{cases} \quad (17)$$

Herein, m_X denotes the mass of grown yeasts (as dry mass), which increases with time during the process. This biomass is obtained from the current cell concentration multiplied with the suspension volume plus the withdrawn sample volume: $m_X(t) = C_X(t) \cdot (V(t) + \Sigma V_{\text{sample}})$. This mass is the biomass present in a nonsampled process, but not the real biomass in the bioreactor. The unknown growth-limiting factor, U , is most likely a vitamin since supplementation of whey media with yeast extract [60, 61] or vitamins [61–63] stimulated the growth of cultivated *K. marxianus*.

The determination of the $\mu_{\max}$ and $m_{X,\text{crit}}$ parameters was based on the here published batch cultivations. The measured $C_X(t)$ data were at first converted to $m_X(t)$ data, as just explained. The measured $n_{\text{CO}_2}(t)$ data used for the same purpose were corrected in the same way. These $m_X(t)$ and $n_{\text{CO}_2}(t)$ data are depicted in Fig. 3 for the period of respiratory sugar utilization. The switch from the exponential to the linear growth becomes clearly visible for both variables. Further data analysis was carried out with the $n_{\text{CO}_2}(t)$ data because of their higher precision and data point density.

Adaptation of the $\mu(n_{\text{CO}_2}(t))$ model to the $n_{\text{CO}_2}(t)$ data of the exponential growth phase yielded quite similar $\mu_{\max}$ values (Table 1) and resulted in an average of $\mu_{\max} = 0.5426 \pm 0.0073 \text{ h}^{-1}$. This value is well comparable with the $\mu_{\max}$ data for *K. marxianus* DSM 5422 measured under similar conditions [17, 46]. In highly diluted DW basic medium, *K. marxianus* DSM 5422 grew 10% faster ($\mu_{\max} = 0.600 \pm 0.005 \text{ h}^{-1}$ [11, 12, 46]) as in nondiluted DW basic medium, since the high content of sugar and minerals in the latter inhibits growth slightly. The inhibitory effect of sugar, salts, and whey-borne minerals on the growth of *K. marxianus* was studied in [53, 64, 65].

A second model adaptation to the $n_{\text{CO}_2}(t)$ data (Fig. 3B) was aimed at determining the individual t_{crit} values for each experiment. This model combined exponential growth at $t \leq t_{\text{crit}}$ with linear growth at $t > t_{\text{crit}}$, using individual $\mu_{\max}$ values, as just identified (Table 1). Model adaptations were restricted to intervals of respiratory growth ($RQ \approx 1 \text{ mol/mol}$), as the respiration-fermentative metabolism results in extra CO_2 formation. The obtained t_{crit} values are similar for the eight cultivations (Table 1) and yield an average value of $13.69 \pm 0.22 \text{ h}$. The individual t_{crit} values correspond to the respective $n_{\text{CO}_2}(t_{\text{crit}})$ values where the exponential growth turns to linear growth. The individual $n_{\text{CO}_2}(t_{\text{crit}})$ values were similar and gave an average of $n_{\text{CO}_2}(t_{\text{crit}}) = 0.129 \pm 0.004 \text{ mol}$. The t_{crit} values also correspond to the respective $m_{X,\text{crit}}$ values that were determined from the $m_X(t)$ data via interpolation, resulting in similar data (Table 1) and yielding an average value of $m_{X,\text{crit}} = 3.75 \pm 0.13 \text{ g}$. This average $m_{X,\text{crit}}$ value corresponds to a critical cell concentration of approximately 6.25 g/L (calculated for $V = 0.6 \text{ L}$).

When yeast growth switches from the exponential to a linear mode, the specific growth rate decreases with typical kinetics (Fig. 1D), which inevitably influences the intracellular iron content, x_{Fe} (compare Eq. (8)).

Figure 3. (A) Formed biomass, and (B) formed carbon dioxide during mixed exponential and linear growth during the aerobic batch cultivation of *K. marxianus* DSM 5422 in a 1-L stirred bioreactor using 0.6 L DW basic medium. The composition of the medium was slightly varied regarding available iron, but the unknown compound *U* was the only growth-limiting factor in the depicted period; cultivation conditions as stated in the legend of Fig. 1. Adverse effect of sampling was taken into account by calculation. Full lines represent model calculations for a switch from the exponential to the linear growth at t_{crit} , while the dashed lines depict fully exponential growth without such a switch. Time scales of individual experiments have been transformed with the aim of unification by subtracting individual t_{crit} values from process time t .

The yield coefficients of yeast growth, $Y_{X/S}$, for *K. marxianus* DSM 5422 were determined as well (Table 1). The $Y_{X/S}$ values were calculated from the formed biomass and utilized sugar over the total process. Computation of $Y_{X/S}$ occurred from measured $C_X(t)$ and $C_{S,L}(t)$ data taking the difference between $C_{S,L}(t)$ and $C_S(t)$, volume changes according to Eq. (4), and losses of biomass and sugar by sampling into account. The individual $Y_{X/S}$ values (Table 1) delivered an average yield of $Y_{X/S} = 0.3643 \pm 0.0076$ g/g that is similar to the earlier measured $Y_{X/S}$ data [17,46]. Cultivation of *K. marxianus* DSM 5422 in highly diluted DW medium at moderate temperatures and low ester concentrations delivered a somewhat higher yield of $Y_{X/S} = 0.414 \pm 0.004$ g/g [11, 12]. The slightly diminished $Y_{X/S}$ value in DW basic medium is attributed to a suboptimal medium composition having already been discussed for the μ_{max} parameter. The published $Y_{X/S}$ coefficients for *K. marxianus* under aerobic cultivation conditions in whey-borne media have already been thoroughly considered previously [12].

5.2 Adsorption and precipitation of iron

The x_{Fe} and ψ variables are determined by balancing the iron based on the measured dissolved iron concentrations (Eqs. (9) to (14)). This concept only works as long as iron has not been adsorbed at the cell surface or precipitated by chemical reactions, since these two processes would pretend microbial iron uptake and result in inaccurate x_{Fe} and ψ data. The same problem arises when iron uptake is measured directly by detecting an incorporation of ^{55}Fe or ^{59}Fe isotopes into the biomass [32,36,47,48,50,51].

Iron was applied as ferrous sulfate but Fe^{2+} ions are quickly oxidized by molecular oxygen to Fe^{3+} ions. Fe^{3+} ions are not stable at higher concentrations and tend to form insoluble ferric oxide-hydroxide, $\text{FeO}(\text{OH})$ [66,67]. The solubility coefficient of $\text{FeO}(\text{OH})$ is approximately 10^{-37} [68], that is the solubility in

water is very low and most of the ferric iron precipitates. The higher the pH is, the lower the solubility of ferric iron is. A pH of 5 in the growth period and even higher values of up to pH 7.6 after sugar depletion let us therefore expect a nearly complete precipitation of iron. Surprisingly, no significant changes in the dissolved iron concentration were observed from 24 to 36 h (Fig. 2), where the pH markedly increased. An analysis of the biomass at the end of cultivation regarding adhered iron (Section 4.3) confirmed that iron was neither adsorbed nor precipitated in significant amounts (0.7 $\mu\text{g/g}$ on average that is low compared to the intracellular iron content (Table 2)).

The absence of iron precipitation despite pH 7.6 at the end of cultivation is explained by the complexing effect of citrate. DW basic medium contains approximately 1.5 g/L citrate that originates from milk and is not assimilated by the yeasts [14]. Citrate is an iron chelator where the structure of formed complexes depends on the pH and the molar iron–citrate ratio [69]. Whey-borne citrate obviously avoids iron precipitation but could also influence microbial iron uptake (see the details below).

5.3 Iron content of biomass

The intracellular iron content of the biomass, x_{Fe} , represents the microgram of intracellular iron per gram of biomass given as the dry weight. The x_{Fe} parameter was calculated from repeatedly measured $C_{Fe,L}$ data (Section 3.3). The reliability of this method has been proven (Section 5.2).

During the exponential growth phase, the iron uptake is equal to the intracellular dilution of iron during growth ($\psi = \mu_{max} \cdot x_{Fe}$), and the x_{Fe} values thus did not temporally change (compare Eq. (8)). The individual x_{Fe} values of this period were similar in all eight experiments (Fig. 4, Table 2) and yielded an average of $x_{Fe} = 134.4 \pm 5.6$ $\mu\text{g/g}$.

Table 2. Parameters of iron uptake during the aerobic batch cultivations of *K. marxianus* DSM 5422 in a 1-L stirred bioreactor using 0.6 L DW basic medium supplemented with trace element solution resulting in different initial iron concentrations

Parameter	Cultivation process							
	A	B	C	D	E	F	G	H
Iron, supplied during the process [mg]	0.000	0.000	0.000	0.000	2.775	2.777	2.777	2.772
Iron, initially present, $C_{Fe,L}(t=0)$ [mg/L]	2.167	3.211	4.240	5.325	2.145	3.250	4.284	5.342
Iron, available in total [mg]	1.305	1.936	2.559	3.217	4.046	4.737	5.363	6.000
Average x_{Fe} at exponential growth [$\mu\text{g/g}$]	122.3	134.3	135.4	132.5	151.2	135.4	128.8	135.6
Average ψ at exponential growth [$\mu\text{g}/(\text{g}\cdot\text{h})$]	75.0	72.3	71.6	69.7	76.4	76.0	68.1	69.3
x_{Fe} after sugar depletion [$\mu\text{g/g}$]	82.7	132.7	163.2	195.3	206.5	184.9	190.1	179.5
x_{Fe} after 24 h of cultivation [$\mu\text{g/g}$]	77.0	119.5	155.9	195.4	208.2	188.9	189.6	175.9
x_{Fe} after 36 h of cultivation [$\mu\text{g/g}$]	74.2	115.0	145.5	177.5	197.1	183.5	176.9	172.1

Note: Processes without (A–D) or with (E–H) a feed of iron stock solution are as explained in Fig. 2. Cultivation conditions are as stated in the legend of Fig. 1. Parameters are derived from measured dissolved iron concentrations by balancing the mass of iron.

Figure 4. Intracellular iron content of the biomass, x_{Fe} , during the aerobic batch cultivation of *K. marxianus* DSM 5422 in a 1-L stirred bioreactor using 0.6 L DW basic medium supplemented with trace element solution resulting in different initial iron concentrations; (A) processes without or (B) with a feed of iron stock solution ($F_{Fe} = 1.21 \text{ mL/h}$ from 14 to 21 h, labeled by the gray area); cultivation conditions as stated in the legend of Fig. 1.

In the subsequent linear growth phase (Fig. 3), the growth rate steadily decreased and the dilution of the intracellular iron continuously lessened. This slowed-down dilution combined with a continued iron uptake should result in a temporarily increasing x_{Fe} value since $\mu \cdot x_{Fe}$ is smaller than ψ , and dx_{Fe}/dt becomes positive. Such x_{Fe} increase during linear growth ($t > 14 \text{ h}$) is observed in most experiments (Fig. 4). However, this x_{Fe} increase is restricted by two factors: (1) a limited availability of iron in the surrounding medium, and (2) a maximum iron storage capacity of yeasts.

Continued iron uptake diminishes the dissolved iron concentration and can result in iron depletion (Fig. 2), which, in turn, stops iron uptake. Such iron depletion is observed in experiments without an iron feed, resulting in an x_{Fe} decrease due to continued yeast growth (Fig. 4A; then $dx_{Fe}/dt = -\mu \cdot x_{Fe}$). This x_{Fe} reduction ended after the growth stopped. The moment, where the x_{Fe} increase switched to an x_{Fe} decrease, depended on the initially available iron; the lower the $C_{Fe,L}(t=0)$ was, the earlier this switchover happened. In experiments with an iron feed, dissolved iron did not limit iron uptake, so that x_{Fe} approached the maximum storage capacity, $x_{Fe,max}$ (Fig. 4B).

The x_{Fe} parameter approached $x_{Fe,max}$ only in five experiments (the four tests with an iron feed, and the one test without an iron feed but with the highest initial $C_{Fe,L}(t=0)$ value). The single maximum x_{Fe} values (Table 2) were used to calculate an average of $x_{Fe,max} = 192 \pm 10 \mu\text{g/g}$ being valid for $t = 24 \text{ h}$ where the iron

uptake finished. Lesuisse et al. [50] cultivated *S. cerevisiae* in a glucose medium with surplus iron and received a much higher intracellular iron content of ca. $2300 \mu\text{g/g}$ for unknown reasons.

The biomass formation was nearly identical in all experiments (Table 1), which means that yeast growth was not restricted by iron. Such a restriction only occurs at a severe iron limitation, whereby the x_{Fe} value approaches $x_{Fe,min} \approx 7 \mu\text{g/g}$ [14, 17].

5.4 Specific iron-uptake rate

The time-dependent biomass-specific iron-uptake rates of the yeasts, ψ , were calculated from the temporarily changing iron content of the yeasts, $x_{Fe}(t)$, and the specific growth rates, $\mu(t)$, using Eq. (14). Here, the iron-uptake rate was determined under growth conditions in a real cultivation process but not in an artificial environment, as usual (e. g. iron absorption measured in short-term tests using resting cells [32, 35–38, 50]).

The obtained iron-uptake rates are depicted versus the process time (Fig. 5A and B) and the iron content of biomass (Fig. 5C and D). The ψ data fluctuate distinctly due to significant error propagation at calculation, but nevertheless provide a deeper insight into iron uptake by *K. marxianus* DSM 5422.

During exponential growth with μ_{max} , an equilibrium exists between the iron uptake into the cells and the intracellular dilution of iron under growth conditions with the result that x_{Fe} does

Figure 5. Specific iron-uptake rate, ψ , depending on (A, B) time or (C, D) the intracellular iron content, x_{Fe} , during the aerobic batch cultivation of *K. marxianus* DSM 5422 in a 1-L stirred bioreactor using 0.6 L DW basic medium supplemented with trace element solution resulting in different initial iron concentrations; (A, C) processes without or (B, D) with a feed of iron stock solution ($F_{Fe} = 1.21$ mL/h from 14 to 21 h); cultivation conditions as stated in the legend of Fig. 1.

not change in this period, so that Eq. (8) yields $\psi = \mu_{max} \cdot x_{Fe}$. The ψ values were calculated individually for the eight experiments (Table 2). These single ψ values are very similar, as expected, and resulted in an averaged value of $\psi(\text{exp. Phase}) = 72.3 \pm 2.2 \mu\text{g}/(\text{g} \cdot \text{h})$. A nearly identical value is obtained by multiplying the average μ_{max} value with the average x_{Fe} value in the exponential phase: $0.5426 \text{ h}^{-1} \cdot 134.4 \mu\text{g/g} = 72.9 \mu\text{g}/(\text{g} \cdot \text{h})$. This ψ value is valid till $t_{crit} \approx 13.7$ h, and is depicted in Fig. 5A and B as a straight line and symbolized in Fig. 5C and D with a large circle.

In the linear growth period, the specific growth rate diminishes (Fig. 1D), the iron uptake exceeds the iron dilution during growth, and x_{Fe} temporarily increases (Fig. 4). Continued iron uptake, however, requires available dissolved iron, which is assured without exception only in the feed experiments. In experiments without an iron feed, the iron is sooner or later depleted (Fig. 2) and, hence, the iron-uptake rate drops to zero (Fig. 5A) and continued growth reduces x_{Fe} (the x_{Fe} data points are running along the x-axis in Fig. 5C). The only exception in this relation is experiment D, where the initially added iron just covered the demand. However, the iron-uptake rate decreased over time even if iron was available in excess (Fig. 5B) since the iron absorption capacity of the yeast cells is restricted because x_{Fe} cannot exceed its maximum value $x_{Fe,max}$ (Fig. 4B). When x_{Fe} approaches $x_{Fe,max}$, ψ converges to zero (symbolized in Fig. 5D by the lower large circle). Iron uptake by *S. cerevisiae* depended also on the iron content of the yeasts; the higher the intracellular iron content was, the smaller the iron-uptake rate became [50].

The mathematical formulation of the iron-uptake rate is based on the research of Geider et al. [70] who modeled the uptake of nitrogen by phytoplankton depending on two

factors: (1) the inorganic nitrogen content of the surrounding medium, and (2) the intracellular nitrogen content of algae. Here, the iron-uptake rate depends on the dissolved iron concentration and the intracellular iron content:

$$\psi = \psi_{max} \cdot \gamma_{\psi}(C_{Fe,L}) \cdot \gamma_{\psi}(x_{Fe}) \quad (18)$$

The ψ_{max} parameter is a maximum ψ value, and the $\gamma_{\psi}(C_{Fe,L})$ and $\gamma_{\psi}(x_{Fe})$ functions consider the effect of the extracellular and intracellular iron concentration, respectively.

Absorption of iron from the surrounding medium by *S. cerevisiae* occurs via low- and high-affinity transporters [29, 31, 33, 34, 71]. Nothing is yet known in this respect for *K. marxianus*. Here, it is assumed that iron absorption occurs exclusively via a low-affinity transporter since most iron was taken up at high dissolved iron concentrations. The $C_{Fe,L}$ -dependent gamma function is formulated as a Monod expression:

$$\gamma_{\psi}(C_{Fe,L}) = \frac{C_{Fe,L}}{C_{Fe,L} + K_{Fe}}$$

This function approaches 1 at a high dissolved iron concentration, while the function becomes zero in iron-depleted medium. The other γ_{ψ} function was formulated following Geider et al. [70]:

$$\gamma_{\psi}(x_{Fe}) = \left(\frac{x_{Fe,max} - x_{Fe}}{x_{Fe,max} - x_{Fe,min}} \right)^n \quad (19)$$

The $x_{Fe,min}$ and $x_{Fe,max}$ parameters denote the minimum and maximum iron content of yeast biomass, respectively, and exponent n determines the degree of nonlinearity of the ψ - x_{Fe}

relation; here, this relation exhibits a progressively declining slope (Fig. 5D) that is obtained for $n < 1$. The $\gamma_\psi(x_{Fe})$ function is maximal at $x_{Fe} = x_{Fe,min}$, and $\gamma_\psi(x_{Fe})$ becomes zero when x_{Fe} approaches $x_{Fe,max}$.

The available $\psi(C_{Fe,L})$ data do not allow a precise K_{Fe} determination since most iron uptake experiments were performed at a high $C_{Fe,L}$ level. The adaptation of the model to the experimental data resulted in an estimated value of $K_{Fe} \approx 200 \mu\text{g/L}$ (Supporting information 2). The $x_{Fe,min}$ parameter was determined earlier and amounted to approximately $7 \mu\text{g/g}$ [14, 17], and $x_{Fe,max}$ was identified to be $192 \mu\text{g/g}$ (see earlier). The maximum iron-uptake rate ψ_{max} and exponent n were estimated from the ψ - x_{Fe} data measured under conditions, where $\gamma_\psi(C_{Fe,L}) \approx 1$; adaptation of the ψ function according to Eq. (18) with $\gamma_\psi(C_{Fe,L}) = 1$ to the data in Fig. 5D yielded $\psi_{max} = 164 \mu\text{g/(g}\cdot\text{h)}$ and $n = 0.7$. The ψ - x_{Fe} function for these parameters is shown in Fig. 5D.

It may be assumed that our findings on iron uptake by *K. marxianus* DSM 5422 are transferable to other *K. marxianus* strains at least in a qualitative manner since iron uptake systems seem to be highly conserved. Kwok et al. [32] studied iron uptake via the Fet3p–Ftr1p transporter system in a wild-type strain of *S. cerevisiae* and obtained a larger ψ_{max} value of approximately $400 \mu\text{g/(g}\cdot\text{h)}$, while measurements of Lesuisse et al. [50] for *S. cerevisiae* resulted in iron-uptake rates that were one to two magnitudes smaller.

Surprisingly, iron was not absorbed completely from the medium even if there was a deficit in iron (compare Figs. 2A and 4A). This residual iron is explained by the presence of approximately 1.5 g/L citrate in DW basic medium [14], which originated from milk and ended up in the used whey. Citrate forms citrate–iron complexes [69] and assumedly competes with the iron transporter for dissolved iron. This competition lastly prevents a complete transporter-mediated iron absorption. The inhibition of iron uptake by the chelating action of citrate has already been described for *S. cerevisiae* [32] and marine phytoplankton [72, 73]. EDTA as another strong iron chelator binds both Fe^{2+} and Fe^{3+} ions intensively [68], so that dissolved iron is no longer bioavailable. Dissolved iron actually suppresses the formation of ethyl acetate by *K. marxianus* and *C. utilis* [14–22], but the supplementation of iron-rich culture medium with EDTA restored the ester synthesis by iron complexation [19]. In iron-starved *K. marxianus* DSM 5422 cells, a high-affinity transport system seems to be induced, which enables a nearly complete absorption of iron from DW basic medium despite the presence of citrate [14, 15, 17].

5.5 Process simulation

Process simulations were performed using the introduced model to check if this model reproduced the real processes (Figs. 1–5). Some additional model equations, the used model parameters, and the simulation results are given in the Supporting information 2. The simulated curves represent the measured data quite well confirming the validity of the established model.

The created model can be used for making predictions of processes that have not yet been studied experimentally. The model is extensible by model equations for describing the synthesis of

ethyl acetate from lactose taking place at low x_{Fe} values. There are enough data available for this model extension [11–17, 42] but would go beyond the scope of the here presented study. After such an extension, the model should be a valuable tool for model-based development and optimization of processes for the biotechnological production of ethyl acetate from whey or other renewable resources.

5.6 Outlook

Ethyl acetate is an important organic solvent with countless applications such as for chemical reactions, cleaning surfaces, processing coating formulations, extraction and chromatographic recovery of pharmaceuticals, and for the production of adhesives, print colors, and other paints. The estimated annual world production of this ester amounts to 1.7 million tons [4]. The current production of ethyl acetate proceeds via petrochemical processes and is solely based on fossil resources such as natural gas and crude oil constituents, whereby acetic acid, ethanol, ethylene, and/or acetaldehyde are intermediates [5, 74–76].

The microbial synthesis of ethyl acetate from sugar by *K. marxianus*, *C. utilis*, *W. anomalus*, and other yeasts [5] could be an interesting alternative. These yeasts exhibit yields ranging from 0.02 to 0.29 g ethyl acetate per gram consumed sugar depending on the conditions, while the theoretical maximum ester yield for glucose amounts to 0.515 g/g [5]. The conversion of whey-borne lactose to ethyl acetate with *K. marxianus* DSM 5422 was best under strict iron limitation and at an elevated temperature of 42°C ($Y_{EAS} = 0.29 \text{ g/g}$ [11]).

The here presented experiments were performed with whey-based medium but ethyl acetate could also be produced from other feedstocks such as sugar beet, sugar cane, and sorghum or from sugar-rich agricultural waste (e.g. from pineapple processing). Starch-rich feedstocks such as wheat and maize are also useable after saccharification. Generally, all raw materials being employed for fermentative ethanol production can also be applied to the considered process. The supplements needed for the microbial conversion process are highly determined by the used feedstock (depending on individual nutrient deficits) and are assessable from the composition of the respective feedstock and the requirements of the yeasts [46].

Upscaling of the proposed process has already been successfully performed in a 70-L stirred reactor [15]. The volatility of the ester enables the process-integrated recovery of ethyl acetate by stripping and subsequent separation from the exhaust gas [12, 16, 42]. Such a method of product recovery improves the economy of the total process and allows for a selective ester separation (besides ethyl acetate, only some ethanol is stripped). Future development of an innovative membrane-based separation process should further improve both the economy and selectivity of product recovery.

The here presented experimental studies of iron uptake by *K. marxianus* were mathematically formulated. This partial model will be later implemented in an overall model describing the total process of microbial ester synthesis, determined by the level of intracellular iron. Such an overall model finally allows for a parameter optimization and technical engineering

of a large-scale process for ester production based on whey and other renewables.

Nomenclature

C_{Fe}	[g/L]	Concentration of dissolved iron related to the suspension volume
$C_{Fe,L}$	[g/L]	Concentration of dissolved iron in the aqueous phase
$C_{Fe,L,0}$	[g/L]	Concentration of dissolved iron in the supplied iron stock solution
C_S	[g/L]	Concentration of sugar related to the suspension volume
$C_{S,L}$	[g/L]	Concentration of sugar in the aqueous phase
C_X	[g/L]	Concentration of biomass in the cell suspension, given as dry weight
F_{Fe}	[L/h]	Flow rate of the supplied iron stock solution
F_{Ev}	[L/h]	Flow rate of evaporated water discharged with the exhaust gas
F_{pH}	[L/h]	Feed rate of the pH corrective (regulated by the pH controller)
$F_{Sampling}$	[L/h]	Rate of the suspension sampling (discrete events described as a continuous process)
K_{Fe}	[g/L]	Half-saturation constant of iron uptake
K_S	[g/L]	Half-saturation constant of growth with lactose
m_{Fe}^{int}	[g]	Mass of intracellular iron within the balance space
m_{Fe}^{ext}	[g]	Mass of extracellular iron within the balance space
m_{Fe}^{total}	[g]	Mass of total iron within the balance space
m_X	[g]	Mass of biomass given as dry weight
$m_{X,crit}$	[g]	Critical mass of biomass given as dry weight
n	[–]	Exponent of the $\gamma_\psi(x_{Fe})$ function
n_{CO_2}	[mol]	Amount of formed carbon dioxide as a cumulative value
n_{O_2}	[mol]	Amount of consumed oxygen as a cumulative value
OD_{600nm}	[–]	Optical density measured at 600 nm
RQ	[mol/mol]	Respiratory quotient
t	[h]	Process time
t_{crit}	[h]	Moment where exponential growth turns to linear growth
V	[L]	Volume of the cell suspension
V_L	[L]	Volume of the aqueous phase
V_{X*}	[L]	Volume of intact biomass
x_{Fe}	[g/g]	Intracellular iron content of biomass (related to dry weight)
$x_{Fe,max}$	[g/g]	Maximum intracellular iron content of biomass
$x_{Fe,min}$	[g/g]	Minimum intracellular iron content of biomass
x_U	[g/g]	Intracellular content of an unknown growth-limiting factor
$Y_{X/S}$	[g/g]	Yield of biomass related to the sugar consumed
$Y_{X/X*}$	[g/g]	Dry mass fraction of biomass (dry mass related to wet mass)

Greek symbols

γ_μ	[–]	Gamma function for describing μ depending on various factors
γ_ψ	[–]	Gamma function for describing ψ depending on various factors
Δt	[h]	Time interval
μ	[h ^{–1}]	Specific growth rate
μ_{max}	[h ^{–1}]	Maximum specific growth rate
ρ_{X*}	[g/L]	Density of intact yeast biomass
φ_{X*}	[L/L]	Volume fraction of intact yeast biomass in the suspension
ψ	[g/(g·h)]	Specific iron-uptake rate (related to dry weight)
ψ_{max}	[g/(g·h)]	Maximum specific iron-uptake rate

Practical application

Whey arising in huge amounts during milk processing in the dairy industry is a valuable renewable resource in the field of White Biotechnology. One application consists of the microbial conversion of whey-borne lactose into ethyl acetate, used as an environmentally friendly solvent. The nonconventional yeast *Kluyveromyces marxianus* is predestinated for such a process due to its ability to form ethyl acetate as a bulk product from lactose at a high rate. Ester synthesis in this yeast is initiated by low intracellular iron content. The intracellular iron level in turn is decreased by cell growth but increased by iron uptake. Hence, the kinetics of iron uptake is of great practical importance for understanding and controlling the process of ester synthesis.

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