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Use of continuous lactose fermentation for ethanol production by *Kluveromyces marxianus* for verification and extension of a biochemically structured model

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HIGHLIGHTS

- Continuous chemostat fermentations of lactose to ethanol have been performed.
- The process was modelled with a biochemically structured approach.
- True yields of the process have been theoretically derived.
- Lactose to ethanol fermentation has been characterized.

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ABSTRACT

A biochemically structured model has been developed to describe the continuous fermentation of lactose to ethanol by *Kluveromyces marxianus* and allowed metabolic coefficients to be determined. Anaerobic lactose-limited chemostat fermentations at different dilution rates (0.02–0.35 h^{-1}) were performed. Species specific rates of consumption/formation, as well as yield coefficients were determined. Ethanol yield (0.655 C-mol ethanol*C-mol lactose^{-1}) was as high as 98% of theoretical. The modeling procedure allowed calculation of maintenance coefficients for lactose consumption and ethanol production of $m_s = 0.6029$ and $m_e = 0.4218$ (C-mol) and (C-mol h)$^{-1}$, respectively. True yield coefficients for biomass, ethanol and glycerol production were calculated to be $Y^{true}_{sx} = 0.114$, $Y^{true}_{ex} = 0.192$ and $Y_{sg} = 2.250$ (C-mol) and (C-mol)$^{-1}$, respectively. Model calculated maintenance and true yield coefficients agreed very closely with those determined by regressions of the experimental data. The model developed provides a solid basis for the rational design of optimised fermentation of cheese whey.

1. Introduction

Lactose conversion into ethanol can be achieved through anaerobic fermentation carried out by a suitable microorganism (Berruga et al., 1997). This process has been considered for a long time (Rogosa et al., 1947; Whittier, 1944) as an alternative to dispose of (and valorize) the main waste from the dairy industry, namely cheese whey, which contains ca. 5% (by weight) lactose. Several studies concerning lactose fermentation have been performed (Ghaly and El Tawel, 1994; Ozmihci and Kargi, 2007a; Sansonetti et al., 2009, 2010, 2011). Most of the studies presented in the literature involve batch processes (Grubb and Mawson, 1993; Kourkoutas et al., 2002; Sansonetti et al., 2011; Silveira et al., 2005). Nevertheless, the possibility to operate in continuous mode has been considered by several authors, using different reactor configurations (Cheryan and Mehaia, 1983; Linko et al., 1981; Ozmihci and Kargi, 2008, 2007b).

Modelling of the fermentation process has been considered in several studies. They deal mainly with kinetic studies of the reactions involved (Dourado et al., 1987; Lee et al., 1983). The main criticism of these models is the empirical nature of the equations involved. Indeed, no physical meaning can be attributed to the many terms constituting the model, and this limitation results in poor descriptive and predictive performance. In other words, these empirical models do not allow a complete understanding of the real phenomena going on behind what is directly observable, therefore, a knowledge-based approach would be more appropriate. An interesting alternative, that can be adopted to model a

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Nomenclature

K	metabolic coefficient for biomass polymerization, [mol ATP∗(C-mol bio)$^{-1}$]
m	maintenance coefficient, [mol ATP∗(C-mol biom.∗h)$^{-1}$]. Subscripts s and e in the text are referred to lactose and ethanol, respectively.
q_i	volumetric reaction rate of species i, [C-mol (L h)$^{-1}$]
r_i	specific reaction rate of species i [C-mol (g DW h)$^{-1}$]
R_{ATP}	ATP consumption rate [mol ATP (L h)$^{-1}$]
t	time [h]
X	biomass concentration [C-mol L^{-1}]
Y_{ij}	yield of species j on species i, [C-mol J (C-mol i)$^{-1}$]
Y_{ATP}	ATP yield, [mol ATP (C-mol biom.)$^{-1}$]

Subscripts

e	ethanol
g	glycerol
s	substrate (lactose)
x	biomass

Greek letters

δ	ATP consumption due to biomass precursors formation [mol ATP (C-mol biom.)+]
δ_x	amount of carbon lost as CO_2 in the biomass production process [(C-mol) (C-mol)$^{-1}$]
μ	specific biomass growth rate, [h^{-1}]

fermentation process, is the so called "biochemically structured approach" which was introduced for the first time by Roels (1980, 1983). It is based on the consideration of the main metabolic pathways in the working microorganism, namely anabolic, catabolic, polymerization and maintenance reactions; its particular advantages will be illustrated later on. Several examples of this approach are found in the literature where the same principles have been applied to different processes such as sucrose consumption (Krzystek and Ledakowicz, 1997), aerobic growth of *Kluyveromyces. fragilis* on lactose (Krzystek and Ledakowicz, 2000) and poly-(b-hydroxybutyrate) (PHB) production (Heijnen et al., 1979). For a review of the assumptions and results in these works, see Sansonetti et al. (2011). Although this approach has given good results yielding deep insights into the processes to which it has been applied, it had never been used for anaerobic fermentation of lactose until recently (Sansonetti et al., 2011). Furthermore, given there are only a few industrial applications of the lactose-to-ethanol process (all in batch configuration), namely *Carbery Group* (Ireland), *Anchor Ethanol* (New Zealand) and *Golden Cheese* (USA) it can be concluded that there is considerable scope for further efforts towards proper modelling aimed at a better characterization and optimization of the process.

Recently we described a new biochemically structured model for lactose fermentation to ethanol by *Kluyveromyces marxianus* (Sansonetti et al., 2011), which can be considered the microorganism of choice for this process (Grubb and Mawson, 1993; Silveira et al., 2005). However, batch fermentation data was used and the assumptions of pseudo steady state were necessary. Furthermore, unsterile ricotta cheese whey was used as substrate. In order to properly characterize the model, chemostat cultivations in a simple sterile medium are to be preferred. In the present work, chemostat fermentations of lactose to ethanol by *K. marxianus* have been studied at different dilution rates to generate a set of data. The data set was used to check the applicability of our previous biochemically structured model and calculate key metabolic coefficients to estimate process yields. Such yields estimated through a knowledge-based approach would imply two particular advantages, that is the possibility to perform an accurate design of the bio-process, and to gain a deep insight into the hidden metabolic phenomena related to the process. The resulting model is meant to be an additional tool for rational design and scale-up of the process in this configuration and also in other configurations such as fed-batch.

2. Theoretical background and modelling

2.1. Theoretical background

The biochemically structured approach is based on the main metabolic pathways involved in the carbon flux from the substrate to the products and was introduced by us for *K. marxianus* recently (Sansonetti et al., 2011). Basically, these processes can be divided into: (1) anabolic reactions, in which the formation of biomass precursors occurs, usually with the consumption of a certain amount of energy (ATP). However, TCA cycle intermediates are necessary in anabolic reactions (e.g. as amino acid precursors) but the reducing equivalents produced cannot be regenerated due to the inoperative respiratory chain under anaerobic conditions, which also leads to a net overproduction of reducing equivalents (indicated as NADH but representing all the forms of reducing equivalents). (2) Catabolic reactions, in which ATP is produced at the expense of a certain amount of substrate. (3) Polymerization of biomass precursors into active biomass with an extra consumption of ATP and (4) maintenance-associated ATP consumption. In order to identify the particular reactions involved in lactose fermentation, it is necessary to consider the metabolic structure involved. Lactose is mainly converted into biomass, ethanol, glycerol, carbon dioxide and acetaldehyde. The main metabolic pathways are depicted in Fig. 1. It should also be noted that the excreted acetaldehyde is always present in negligible amounts (Nielsen et al., 2003), therefore it will not be considered in development of the model. Two other processes that play important roles in the fermentation reactions should also be considered in the model, namely polymerization of biomass precursors and biomass maintenance. With lactose as the sole carbon source, the stoichiometry of each metabolic pathway is given in Table 1.

As we have explained previously (Sansonetti et al., 2011), ATP consumption for both polymerization and maintenance results in a net ATP consumption rate, R_{ATP} [(mol ATP)∗(L∗h)$^{-1}$], which can be written as the sum of two terms, one growth-associated (IV), proportional to q_x (the rate of formation of biomass expressed as (C-mol *biomass*)∗(L∗h)$^{-1}$), and one due to the maintenance (V). ATP consumption for maintenance is assumed to be directly related to the biomass concentration X [(C-mol) L^{-1}] (Stouthamer and Bettenhaussen, 1973) as shown in Eq. (1) where m [(mol ATP) (C-mol biomass h)$^{-1}$] represents the maintenance coefficient for biomass and K [(mol ATP) (C-mol biomass)$^{-1}$] is the metabolic coefficient indicating the energy needed for biomass polymerization.

$$R_{ATP} = -Kq_x - mX \quad (1)$$

Process I contains two metabolic coefficients, δ_x [(C-mol lactose) (C-mol biomass)$^{-1}$] that represents the amount of carbon lost as carbon dioxide, and δ [(mol ATP) (C-mol biomass)$^{-1}$], which is the amount of ATP consumed in the anabolic formation of biomass precursors. In general this term depends on the culture conditions. The stoichiometric coefficients and their relationships in the processes reported in Table 1 come from the application of the concept of "element balance" during anaerobic growth of the microorganism with product formation. For further insights on this derivation the original text for this concept can be consulted (Roels, 1980).