THE EFFECT OF THE ELECTRIC FIELD ON LAG-PHASE, ETHANOL AND β-GALACTOSIDASE PRODUCTION OF A RECOMBINANT S. CEREVISIAE GROWING ON LACTOSE

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Abstract. The production of ethanol and β-galactosidase from cheese whey and other sub-products from industries can be a good way of minimizing environmental problems and producing valuable products from low-cost raw materials. A recombinant *S. cerevisiae* NCYC869-A3/pVK1.1 flocculent strain expressing the lacA gene (coding for β-galactosidase) of *Aspergillus niger* under ADHI promotor and terminator was used in the experiments, once this strains has higher ethanol and β-galactosidase productivities. Batch culture experiments were performed in SSlactose medium with 50 g/L lactose. A 2 dm^3 bioreactor with agitation, temperature and pH measurement and control, was used. The experiments were conducted under aerobic and anaerobic conditions. The temperature was maintained at 30 ◦C and the pH at 4.0. Moreover, electrodes were placed inside the bioreactor and experiments were conducted at three different electric fields, ranging from 0.5 to 2.0 $V.cm^{-1}$ to determine the effect of the electric field in the fermentation profile. For all the experiments the biomass, protein, β-galactosidase activity, lactose, glucose, galactose and ethanol were measured. Finally, the lag phase and specific growth rate were calculated. Significant changes in lag phase and biomass yields were found when using $2.0V.cm^{-1}$. The results show that ohmic heating enhances early stages of the fermentation, indicating that ohmically heated fermentations may be extremely useful in food industry.

Keywords: *β*-galactosidase, electric field, growth parameters.

1. Introduction

People that are lactose-intolerant cannot eat several milk and milk-derived products. When the intestine produces little or no lactase, lactose (the milk sugar) is not digested and moves into the colon, where bacteria ferment it, producing hydrogen, carbon dioxide and organic acids. The results of this fermentation are diarrhea, flatulence (gas) and abdominal discomfort. β-Galactosidase (EC 3.2.1.23) is able to cleave β-linked galactose residues from various compounds and is commonly used to cleave lactose into galactose and glucose. β-Galactosidase preparations are widely used for hydrolysis of lactose in milk, milk products, and whey (Richmond, 1981; Gekas and López-Leiva 1985; Fiedurek, 1996).

Domingues et al (2000, 2002) have previously reported that cheese whey permeate could be used as substrate for β-galactosidase and ethanol production by this recombinant strain. The production of ethanol and β-galactosidase from cheese whey and other sub-products from industries can be a good way of minimizing environmental problems and producing valuable products from low-cost raw materials. Moreover, the downstream processing is simplified due to the cell flocculation characteristics that allow a low-cost separation and purification process, decreasing production expenditure.

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Responses of living cells and biochemicals to electrical signals have been used in biotechnology (Bartlett, 1997), bioanalytical techniques and for medical purposes (Stacey, 2003)). Depending on the intensity of the applied electrical potential and on its time function (constant, pulsed, etc), distinct effects in metabolic processes may occur. Such exogenous stimulation determines the appearance of an electric potential across the membrane which depends on the strength of the external field, cell shape, cell radius and membrane properties. Among the several types of stress reported in literature, thermal stress is the most widely studied. It was shown that thermal treatment of *S. cerevisiae* results in an increased tolerance to ethanol which points out to industrial use of induced cell stress. Electric field promoted stress presents industrial advantages over other types of stress because there is no addition of external chemicals which may increase product separation and waste treatment costs. Effects of electric field (or ohmic heating) on microbial growth kinetics were studied in lactic acid bacteria and some changes on lag phase and bacteriocin production were reported (Cho et al, 1996). However no other studies on the sub-lethal effect of electricity in microorganisms during fermentative processes could be found.

2. Materials and Methods

2.1. Microorganism

The microorganism used in this work was a recombinant Saccharomyces cerevisiae NCYC869-A3/pVK1.1. This is a flocculent strain expressing the lacA gene (coding for β-galactosidase) of Aspergillus niger under ADHI promotor and terminator (Domingues et al , 2002).

2.2. Culture Media

The recombinant yeast was maintained at 4 ºC on slants or at –80 ºC in permanent culture of YNB selective medium (6.7 gL^{-1} yeast nitrogen base (w/o amino acids), 20 gL^{-1} lactose). For fermentation SSLactose medium was used (KH_2PO_4 5 $g.L^{-1}$; $(NH_4)_2SO_4$ 2 $g.L^{-1}$; $MgSO_4.7H_2O$ 0.4 $g.L^{-1}$; yeast extract 2.0 $g.L^{-1}$; lactose 50 $g.L^{-1}$).

2.3. Bioreactor operation

Batch culture experiments were performed using a 2 L bioreactor (Figure 1). All the experiments were conducted with agitation, temperature measurement and control, pH measurement and control. Temperature was monitored using type-K thermocouples, with Teflon coating, placed inside the reactor. The temperature was kept at 30 ± 1 ºC by developing a control program in Labview 7.0 Express, National Instruments. The initial pH was 5.4, which was allowed to drop to 4.0 during the fermentation and then kept at 4.0 ± 0.15 by automatic addition of ammonia solution (30 % v/v). The agitation was made by a magnetic stirrer and the bioreactor was aerated with filtered air at a flow rate of 2.5 vvm. These conditions guarantee a minimum of 20 % of dissolved oxygen, during all stages of the fermentation. Anaerobic conditions were attained by an initial sparging of filtered air and then suppressing the air supply. The vessel and tubing were autoclaved for 30 min at 121 ºC.

Two stainless steel electrodes were placed symmetrically inside the bioreactor with a distance of 7.5 cm between them. Each electrode has a surface area of 65 cm^2.

The electric field, ranging from 0 to 2 $V.cm^{-1}$, was generated by an alternating current source, of 50 Hz.

A data-logger was employed to record continuously and simultaneously current intensity, voltage and temperature.

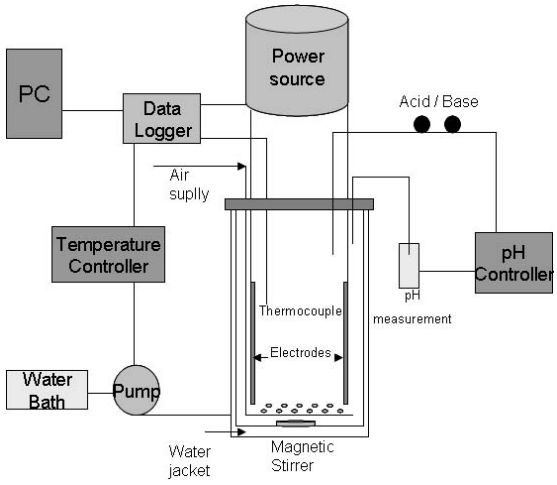

Fig. 1. Schematic diagram of the experimental setup.

2.4. β-Galactosidase activity measurements

β-GAL is an extracelullar enzyme and it was used directly from fermentation broth without further purification. One unit of activity was defined as the amount of enzyme that hydrolyses 1 nmol of p-nitrophenil β-D-galactopyranoside (pNPG) per minute, at 65 °C. The activity was measured as the amount of p-nitrophenol released from pNPG *per* minute. Samples were incubated with 1.7 mM substrate in 0.075 M Na-acetate buffer, pH 4.5. The pH was raised to 10 with 1 M of Na_2CO_3 and the activity was measured spectrophotometrically at 405 nm (Bailey and Linko, 1990).

2.5. Biomass determination

Biomass concentrations were measured as dry-weight (DW) and/or using absorbance methods. The DW was determined by filtering the sample through 0.2-μm filter-paper and then drying at 105 ºC for 24 h. The absorbance was measured at 620 nm and compared to a standard curve for absorbance versus DW previously constructed. Prior to absorbance readings cells were deflocculated with a solution of NaCl (1.5 % w/v, pH 3.0).

2.6. Lactose, glucose, galactose and ethanol measurements

The total reducing sugar (RS) concentration was determined by the dinitrosalicilic (DNS) acid method described elsewhere (Miller, 1959). Sugars (lactose, glucose and galactose) and ethanol were quantified by HPLC (Chrompack, Middleburg, The Netherlands) using a refractive index (RI) detector (JASCO 830-RI Intelligent RI Detector, Jasco, Tokyo, Japan) and a Organic acids column. The eluent was 0.005M H_2SO_4. The

analyses were made at an elution rate of 0.3 ml.min^{-1} and an oven temperature of 60 ºC. The retention times of lactose, glucose, galactose and ethanol were, respectively, 12.2, 14.3, 15.4 and 31.1 min.

3. Results and Discussion

The fermentations were conducted under aerobic and anaerobic conditions and the initial lactose concentration was 50 g.L^{-1}. For both conditions, different electric fields (ranging from 0 to 2 V.cm^{-1}) were applied. Typical fermentation parameters' evolution with time is presented in Figures 2 and 3.

3.1. Aerobic Fermentations

Figure 2 shows that lactose is totally metabolized by the recombinant strain in less than 22 h, when an electric field of 2 V.cm-1 is applied. This represents a reduction of about 6 hours in the fermentation time when comparing to the other aerobic conditions tested and may be due to the increase in the biomass production. In fact, the biomass yield increases linearly with the applied electric field (Figure 4). In fact, the final biomass increases significantly, varying from 7 to 20 g.L^{-1}, with the application of an electric field (Table 1). Stone (cited by Rowley (1972)) also reported that current generated through electrochemical reactions stimulated the growth of bacteria, resulting in bacterial counts 100-fold greater than for controls.

Lag phase was significantly affected by the method of heating (see Table 1), increasing for the lower values of electric field and then decreasing when using higher voltages. In fact, while for an electric field of 2 V.cm^{-1} the lag phase is shorter than for conventional heating, for 0.5 and 1.0 V.cm^{-1} the lag phase increases when compared to a conventional heating (0 V.cm^{-1}) fermentation. The calculation of the growth rate points out to a decrease of that parameter when the lag phase is shorter. Similar results were found by Cho et al (1996) when applying an electric current to lactic acid bacteria. These authors reported significant changes in the lag phase when using ohmic heating during fermentations at sub-optimal temperatures and some minor changes when operating near the optimal temperature. As in the present work, the lag phase also increased slightly when using lower voltages and operating near the optimal temperature.

In this study, although the fermentations were performed at the optimal temperature, some significant changes could be found. Probably these changes would be even more evident if operating at sub-optimal conditions.

Additional research is needed to explain the changes observed in the behavior of the lag period under the influence of an electric field. However, the hypothesis that the use of higher electric fields may improve nutrients absorption due to two different phenomena may be advanced. Such phenomena would be: improved transport through the cell flocs and improved transport through the cell membrane due to pore formation or activation of transport proteins (Castro *et al* (2004) reported some influence of electric field in several food processing enzymes). This hypothesis, however, is yet to be proven.

The extracellular β-galactosidase concentration increased during the exponential and stationary phase and the substrate conversion yield on β-galactosidase remained constant for the lower electric fields (0, 0.5, 1) but decreased when using 2 V.cm^{-1}. Further studies on plasmid stability when applying electric field must be performed, however considering that β-galactosidase is a primary metabolite (directly associated with biomass

production) the decrease observed for higher electric fields may be related to the loss of the plasmid. Domingues et al (2004) reported losses of plasmid of about 30 % during aerobic fermentations (in the absence of an electric field) and this means that only 70 % of the viable cells are producing and excreting β-galactosidase.

The oxygen concentration did not significantly affect β-galactosidase production (Domingues et al, 2004). The oxygen concentration should be sufficient to allow the yeast growth. In any case, oxygen limitation has no effect on the final amount of β-galactosidase produced. Domingues et al, (2004) reported that if the oxygen is in excess, an ethanol consumption phase is expected, increasing further the β-galactosidase production after the original carbon source (sugar) has run out. As the promotor used was the short *ADHI*, the ethanol consumption phase had a stimulatory effect on the promotor. This ethanol consumption phase was not observed in any of the fermentations performed in the present work.

The ethanol yield per unit of biomass was, overall, constant for the conditions tested. Some evaporation occurred during the fermentations and all the results were corrected considering evaporation rates. However ethanol losses due to this phenomenon cannot be estimated and that is the main reason for the presence of some oscillating values in the presented profiles.

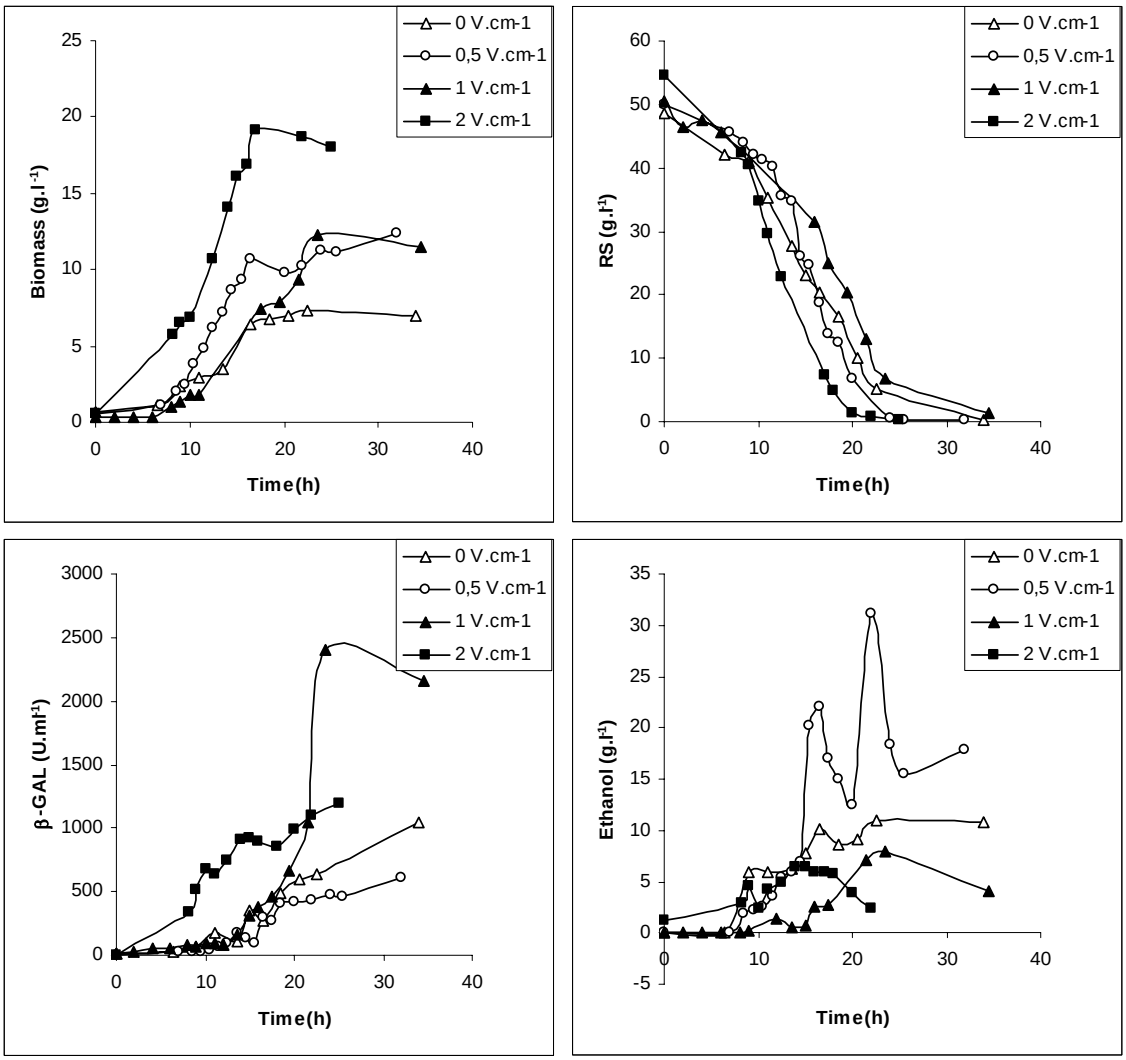

Fig. 2. Aerobic fermentations profile.

3.2. Anaerobic Fermentations

Figure 3 presents the time course for anaerobic fermentations. The analysis of data indicates that the effect of electric field is not as evident as when air is present. Exception made for 2 $V.cm^{-1}$, which takes more then 50 hours to completely metabolize lactose, for the other conditions the fermentation ends at approximately 35 hours.

The biomass yield remains approximately constant (Figure 4) meaning that under anaerobic conditions there is no stimulatory effect of the electric field on growth. This behavior is opposite from the one observed for aerobic conditions but reinforces Rowley‘s statement (1972). This author stated that electrical current will either enhance or inhibit the growth of the microorganisms depending on the experimental conditions. However, there seems to be an increase in the growth rate for lower electric fields (0.5 $V.cm^{-1}$).

As previously observed for aerobic conditions, the substrate conversion yield on β-galactosidase remained constant for the lower electric fields (0, 0.5, 1 $V.cm^{-1}$) but decreased when using 2 $V.cm^{-1}$. This decrease is, once more, probably due to the loss of plasmid therefore stopping the production of β-galactosidase. This also explains the extended fermentation time. Lactose was slowly hydrolyzed in glucose and galactose by the excreted β-galactosidase and this reduced the amount of sugars available for consumption. Domingues et al (2004) reported that under anaerobic conditions the plasmid stability was lower, which can explain the more pronounced effect of reducing β-galactosidase productivity when no air is present.

Ethanol productivity remains, overall, constant.

4. Conclusions

The use of sub-lethal ohmic heating to maintain the temperature during fermentations affected the growth and metabolic activities of this recombinant *S. cerevisiae* growing on lactose. The use of higher electric fields resulted in the shortest lag phase and the maximum biomass yield, under aerobic conditions. When anaerobic conditions were used the lag phase was also significantly reduced but the effect on biomass yields was not so clear.

The use of ohmic heating in industrial fermentative processes may be useful in the early stages of the fermentation (reduction of lag phase); however, further research must be carried out to assess and explain the effect of ohmic heating in the metabolic pathways of microorganisms.

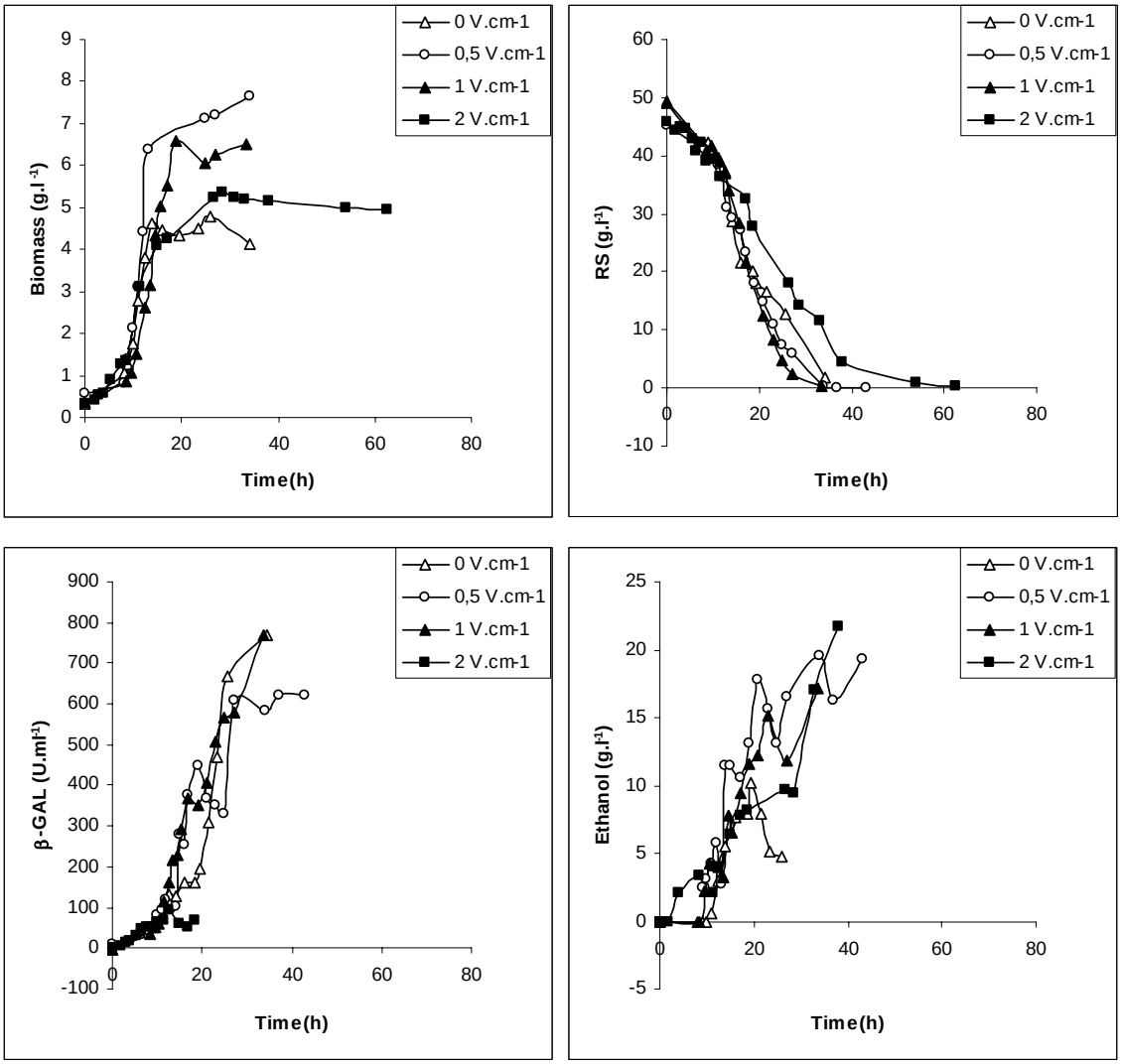

Fig. 3. Anaerobic fermentations profile.

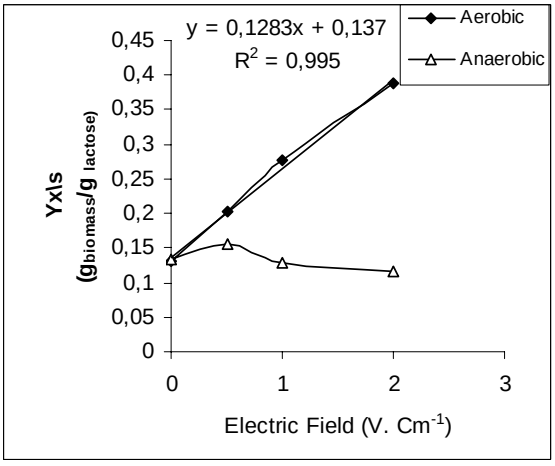

Fig. 4. Biomass yield as a function of electric field .

Table 1. Growth parameters and fermentation yields for the different experimental conditions tested

Electric field ($V.cm^{-1}$)	0		0.5		1.0		2.0	
Kinetic Parameters	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
μ (h^{-1})	0.169	0.291	0.312	0.405	0.209	0,260	0,144	0,176
t_{lag}(h)	3.82	4.15	4.62	6.84	5.83	5,01	0	0.66
t_D (h)	4.10	2.38	2.22	1.72	3.32	2,66	4.81	3.94
Final Biomass ($g.L^{-1}$)	7.02	4.11	10.51	6.52	12.68	6.52	20.23	5.31
$Y_{x/s}$ ($g_{biomass}/g_{lactose}$)	0.153	0.205	0.2026	0.542	0.253	0.102	0.337	0.309
$Y^{G}_{x/s}$ ($g_{biomass}/g_{lactose}$)	0.131	0.133	0.202	0.155	0.276	0.129	0.388	0.300
$Y_{P1/s}$ ($g_{ethanol}/g_{lactose}$)	0.128	0.383	0.286	0.573	0.192	0.432	0.129	0.492
$Y^{G}_{P1/s}$ ($g_{ethanol}/g_{lactose}$)	0.252	0.426	0.306	0.582	0.180	0.373	0.121	0.527
$Y_{P2/s}$ ($U/g_{lactose}$)	1.74×10^4	5.71×10^3	1.10×10^4	1.75×10^4	1.60×10^4	1.22×10^4	2.18×10^4	6.64×10^3
$Y^{G}_{P2/s}$ ($U/g_{lactose}$)	2.13×10^4	1.63×10^4	1.23×10^4	1.73×10^4	2.76×10^4	1.82×10^4	2.13×10^4	7.51×10^3
$Y_{P2/x}$ ($U/g_{biomass}$)	8.07×10^4	3.12×10^4	6.78×10^4	3.05×10^4	$9.1x\ 10^4$	5.5×10^4	1×10^5	3.2×10^4
$Y^{G}_{P2/x}$ ($U/g_{biomass}$)	9.39×10^4	2.0×10^5	5.18×10^4	1.38×10^4	2.0×10^5	9.8×10^4	6.7×10^4	2.5×10^4
$Y_{P1/x}$ ($g_{ethanol}/g_{biomass}$)	0.730	2.43	0.7224	1.411	0.747	1.887	0.391	4.497
$Y^{G}_{P1/s}$ ($g_{ethanol}/g_{biomass}$)	1.652	1.05	1.519	2.692	0.657	2.794	0.287	4.747
qs_{p1} ($g_{ethanol}/g_{lactose}.h^{-1}$)	1.320	0.761	1.091	0.707	1.09	0.603	1.12	0.358
$q^{G}s_{p1}$ ($g_{ethanol}/g_{lactose}.h^{-1}$)	0.670	0.683	1.019	0.695	1.16	0.698	1.19	0.334
qs_{p2} ($U/g_{lactose}.h$)	9.69×10^{-6}	5.11×10^{-5}	$2,84 \times 10^{-5}$	1.10×10^{-5}	1.3110^{-5}	2.14×10^{-5}	6.61×10^{-6}	3.35×10^{-6}
$q^{G}s_{p2}$ ($U/g_{lactose}.h^{-1}$)	7.94×10^{-6}	1.79×10^{-5}	3.21×10^{-5}	3.10×10^{-5}	$7.58x\ 10^{-6}$	1.43×10^{-5}	6.77×10^{-6}	7.00×10^{-6}

5. References

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