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Oxygen effect on batch cultures of *Leuconostoc mesenteroides*: relationship between oxygen uptake, growth and end-products

Abstract Growth and lactose metabolism of a *Leuconostoc mesenteroides* strain were studied in batch cultures at pH 6.5 and 30°C in 10 l modified MRS medium sparged with different gases: nitrogen, air and pure oxygen. In all cases, growth occurred, but in aerobiosis there was oxygen consumption, leading to an improvement of growth yield $Y_{X/S}$ and specific growth rate compared to anaerobiosis. Whatever the extent of aerobic growth, oxygen uptake and biomass production increased with the oxygen transfer rate so that the oxygen growth yield, Y_{X/O_2} , remained at a constant value of 11 g dry weight of biomass/mol oxygen consumed. Pure oxygen had a positive effect on *Leuconostoc* growth. Oxygen transfer was limiting under air, but pure oxygen provided bacteria with sufficient dissolved oxygen and leuconostocs were able to consume large amounts of oxygen. Acetate production increased progressively with oxygen consumption so that the total molar concentration of acetate plus ethanol remained constant. Maximal $Y_{X/S}$ was obtained with a 120 l/h flow rate of pure oxygen: the switch from ethanol to acetate was almost complete. In this case, a 46.8 g/mol $Y_{X/S}$ and a 0.69 h⁻¹ maximal growth rate could be reached.

Introduction

Several investigations (Johnson and McCleskey 1957, 1958; Lucey 1985; Borch and Molin 1989) have definitely showed that *Leuconostoc mesenteroides* are able to assimilate oxygen. Aerobic cultures have better growth yields and better specific growth rates than anaerobic cultures. The end-products are also modified.

In anaerobiosis, these heterolactic bacteria produce mainly D-lactate, ethanol and carbon dioxide (Doelle 1975), but acetate largely substitutes for ethanol in the presence of oxygen (Yashima et al. 1971; Lucey 1985), inducing a production of ATP (Condon 1987), which is directly responsible for an increase of biomass in aerobic cultures compared to anaerobic ones.

Oxygen dissimilation can be considerable but variable among *L. mesenteroides* species (Johnson and McCleskey 1957, 1958). A strong decrease in the amount of dissolved oxygen in spite of effective aeration and agitation led Borch and Molin (1989) to conclude that lactic acid bacteria are able to consume oxygen at a high rate. In addition, oxygen consumption is closely linked to an increase of growth yield and specific growth rate according to the results of Lucey (1985) on 12 *Leuconostoc* strains. Thanks to these authors, oxygen uptake and its direct influence on growth were brought to light.

Our aim in this paper is to define the limits of oxygen tolerance by *Leuconostoc* species. The effects of different gases, from nitrogen (anaerobiosis) to pure oxygen, by the way of air, were tested in batch cultures of a *L. mesenteroides* strain. Instantaneous oxygen consumption was determined with accuracy by the method of gas balance (Cooney et al. 1977). A correlation between oxygen consumption and biomass production could be established. Variation of energetic efficiency Y_{ATP} during each batch culture was also determined.

Materials and methods

Organism

A strain of *Leuconostoc mesenteroides* subsp. *mesenteroides*, a heterofermentative organism, was obtained from a commercial laboratory and identified with an API 50 CH gallery according to Garvie (1986). The strain was stored at -18°C in MRS medium (Biokar Diagnostics) (De Man et al. 1960) with 10% (v/v) glycerol.

Inoculum

A 100 ml sample of liquid MRS medium inoculated with 4 ml *L. mesenteroides* suspension was incubated for 7 h in a 250 ml conical flask at 30°C with magnetic agitation at 250 rpm. The fermentor was inoculated with 0.23% (v/v) of this culture.

Batch cultures

Experimental procedure

A diagram of the apparatus used for batch cultures is shown in Fig. 1. A 15 l fermentor (Applikon) was connected to a bio controller (ADI 1030, Applikon) from which pH and dissolved oxygen tension could be recorded. The pH of the medium was monitored with a heat sterilizable electrode (Ingold). When the pH fell below the set point, the bio-controller activated a pump (Masterflex), which provided the medium with a 5 M ammonia solution to neutralize the acids formed. The dissolved oxygen was measured with a membrane covered polarographic oxygen electrode (Ingold) which was also heat sterilizable. The "zero" was realized by sparging nitrogen into the sterilized medium. When working with air, the full scale was established by sparging air so that a dissolved oxygen concentration of "100%" corresponded to a medium saturated in air. In the case of pure oxygen, the polarographic oxygen electrode was calibrated again so that "100%" corresponded to a medium saturated in pure oxygen. The temperature of the medium was continuously measured and kept constant by a water bath. The stirrer speed was controlled by an independent element (ADI 1020, Applikon). A mass flow meter (Bronkhorst, Hi tec, series F100/200) controlled the inlet gas flow with accuracy. To avoid contamination, a 0.2 µm Teflon filter (Sar torius) was set up at the air inlet.

Fixed parameters

For each batch culture, the temperature was fixed at 30°C, the stirrer speed at 400 rpm, and the pH at 6.5. An anti foam agent was added to the medium, which was saturated with oxygen just before inoculation. A 10-l batch of modified MRS medium (20 g/l glucose was replaced by 40 g/l lactose and Tween 80 was omitted) was sterilized for 55 min at 120°C. Samples were taken every hour during the lag phase and then every half hour.

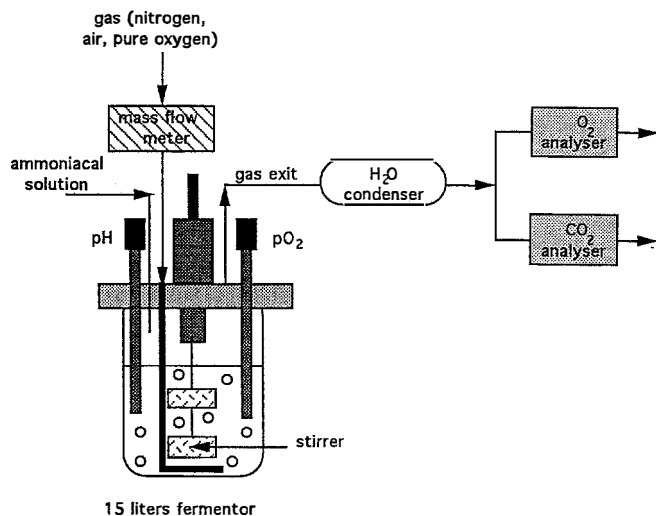


Fig. 1 Experimental set-up for batch cultures and on-line gas measurement

Variables

To test the limits of oxygen uptake by the strain under investigation, the oxygen transfer rate (OTR) to the medium $OTR = k_{La} (c^* - c)$ (mg l⁻¹ h⁻¹), where k_{La} is the volumetric mass transfer coefficient (h⁻¹), c^* is the O₂ solubility (mg/l) and c is the dissolved O₂ concentration, was progressively increased by acting on k_{La} and c^* . k_{La} was first increased by sparging the medium with air and by altering the air flow rate: 60 l/h, 120 l/h and 240 l/h, which corresponded to k_{La} values respectively of 26 h⁻¹, 39 h⁻¹ and 62 h⁻¹. Pure oxygen was then the only way to increase OTR considerably: c^* was increased fivefold. Two flow rates were studied: 36 l/h and 120 l/h. Anaerobiosis was initiated by sparging the medium continuously with a 60 l/h flow rate of nitrogen.

Analysis of the outlet gas

Oxygen uptake and carbon dioxide production were measured on line by means of a paramagnetic gaseous oxygen analyser (570A model, Servomex) and an IR carbon dioxide analyser (series 1400 model, Servomex) respectively. Two cases had to be examined:

On the one hand, when the medium was sparged with air, the general expression of molar oxygen uptake rate given by Cooney et al. (1977) become

$$OUR = (0.266 F_N - P_{O,out} \cdot F_{out})/V \quad (1)$$

where OUR is the oxygen uptake rate (mol l⁻¹ h⁻¹), F_N is the molar flow rate of inert gas (mol/h), $P_{O,out}$ is the partial pressure of oxygen in the outlet gas (atm), F_{out} the molar flow rate of the total outlet gas (mol/h), V is the volume of medium and

$$F_{out} = F_N / (1 - P_{O,out} - P_{c,out})$$

where $P_{c,out}$ is the partial pressure of carbon dioxide in the outlet gas (atm).

A similar expression could be written for the molar carbon dioxide production rate (CPR, mol l⁻¹ h⁻¹) as follows

$$CPR = P_{c,out} \cdot F_{out} / V \quad (2)$$

On the other hand, when the medium was sparged with pure oxygen, Eqns 1,2, based on the conservation of inert gas in the gas flow, became inapplicable. However, since under pure oxygen almost one mole of carbon dioxide was produced for one mole of oxygen consumed (according to our experiments, almost no ethanol was produced), we can write $F_{in} = F_{out}$. In this case, $OUR = (1 - P_{O,out}) \cdot F_{in} / V$ and $CPR = P_{c,out} \cdot F_{in} / V$.

For each inlet gas, instantaneous oxygen uptake [OU(t)] and carbon dioxide production [CP(t)] could then be found by integrating OUR(t) and CPR(t) respectively.

Analyses

The bacterial density of the culture was determined from turbidity measurements at 600 nm. The data were converted to dry weight by means of a calibration graph: dry weight (g/l) = $1.5 \times A_{600}$, and viability was determined by using the epifluorescence method (King et al. 1981). A high-pressure liquid chromatograph (HPLC), with a specific column (Bio Rad Aminex HPX 87H) maintained at 30°C, separated lactose, lactate and acetate. Detection of each component was by means of a refractometer. The mobile phase was 0.005 M sulphuric acid solution with a rate fixed at 0.3 ml/min. The ethanol concentration could also be measured, but for greater accuracy, ethanol was determined by gas chromatography (Chrompack 437A) using a column Chrompack Poraplot Q, wide-bore (0.53 mm × 25 m). The temperature of the heated column was 170°C

whilst the temperature of the injector and detector was 200°C. The carrier gas was nitrogen. A 1% (v/v) isopropanol solution represented the internal standard and was added to samples in the proportion of two volumes of isopropanol for one volume of sample.

Correction of medium volume

pH control caused an increase of the medium volume. Indeed, the error in the volume reached 6.6% when the volume increase was neglected under pure oxygen. In addition, biomass and product concentrations did not refer to the same final volume for each fermentation, since the latter varied with aeration. Considering that the instantaneous medium volume $V(t)$ increased following biomass production, a linear representation was chosen. The two parameters of this model were calculated in order to follow growth evolution closely. Then, for all experimental molar concentration $c_{exp}(t)$ obtained, a correction $c_{corr}(t)$ was applied as follows: $c_{corr}(t) = c_{exp}(t) \cdot V(t)/10$. Finally, each concentration was referred to the same volume (i.e. the initial volume) and comparisons could then be made.

Results

Influence of oxygen on growth

The positive effect of oxygen on growth and oxygen consumption appears clearly in Fig. 2 and Table 1. The more oxygen was provided in the medium, the faster and better was the growth. Growth did occur in anaerobiosis, but when there was dissolved oxygen in the medium, oxygen was consumed and hence there was an increase of biomass production. Oxygen con-

Table 1 Fermentative parameters for each batch culture studied. $Y_{X/S}$ growth yield per mole of sugar, P_X growth productivity (change in biomass with time), μ_{max} maximum specific growth rate, $t_{\mu max}$

Aeration	$Y_{X/S}$ (g/mol)	P_X (g/l/h)	μ_{max} (h ⁻¹)	$t_{\mu max}$ (h)
Anaerobiosis	29.7	0.22	0.45	5
Air, 60 l/h	37.5	0.30	0.62	5.4
Air, 120 l/h	40.3	0.32	0.65	5
Air, 240 l/h	42.1	0.33	0.66	5.4
O ₂ , 36 l/h	44.7	0.39	0.68	6.6
O ₂ , 120 l/h	46.8	0.41	0.69	6.7

sumption occurred after the stationary phase had been reached, although lactose was exhausted, and O₂ was certainly used in cellular maintenance.

A weak aeration (60 l/h) induced a strong increase in growth yield and specific growth rate compared to anaerobiosis; doubling or quadrupling the air flow rate had a slight effect on growth. Strong growth under pure oxygen revealed that *L. mesenteroides* were able to bear pure oxygen. With a pure oxygen inlet rate of 120 l/h, we obtained a 57.6% increase in growth yield and a 53.3% improvement in specific growth rate compared to anaerobiosis; this was considerable. Whatever the aeration, bacteria were all viable when reaching the stationary phase, thanks essentially to pH control, according to the results of Champagne et al. (1989).

A correlation between the total amount of oxygen consumed when biomass production stopped and the final biomass production could be established (Fig. 3). The slope of the linear curve represented the oxygen growth yield and was equal to 11 g dry weight/mole O₂ consumed.

Influence of partial pressure of oxygen in the inlet gas flow

On oxygen consumption

When the medium was sparged with air, oxygen transfer was limiting for growth: the dissolved oxygen concentration fell to zero as soon as the lag phase ended, stayed at zero during the entire exponential phase and rose during the stationary phase (Fig. 4). An increase in air flow rate slightly reduced the limitation by oxygen but did not erase it. Only a strong increase in the partial pressure of oxygen in the inlet gas allowed us to provide the medium with sufficient dissolved oxygen, and the dissolved oxygen concentration did not fall below 4.7 mg/l under pure oxygen; the medium had been saturated in oxygen for the major part of the batch. Thanks to a better oxygen transfer to the medium, oxygen consumption was improved under pure oxygen compared to air.

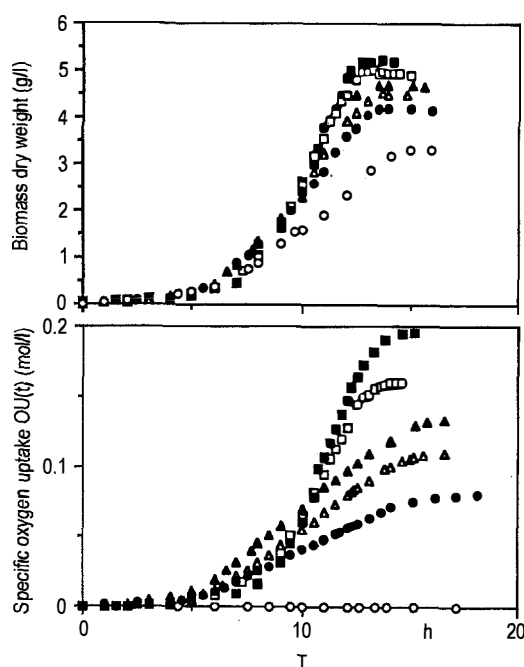


Fig. 2 Growth and specific oxygen uptake for each aeration: ○ anaerobiosis; ● air, 60 l/h; △ air, 120 l/h; ▲ air, 240 l/h; □ O₂, 36 l/h; ■ O₂, 120 l/h

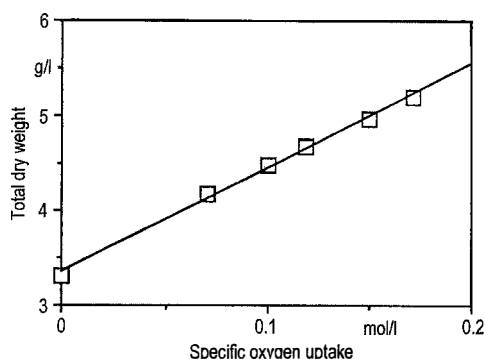


Fig. 3 Correlation between specific oxygen uptake and biomass production for each batch culture

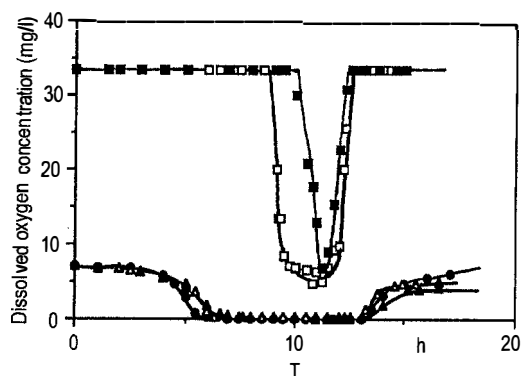


Fig. 4 Dissolved oxygen concentration under ● air, 60 l/h; △ air, 120 l/h; ▲ air, 24 l/h; □ O₂, 36 l/h; ■ O₂, 120 l/h

On specific growth rate

Table 1 gives the time when the maximal specific growth rate was reached. An improvement in specific growth rate with oxygen was accompanied by a delay, which increased with the percentage of oxygen in the inlet gas flow. This delay should correspond to the start of an aerobic metabolic pathway that needed the activation of enzymes.

Influence of oxygen consumption on metabolic end-products of lactose

Among the four end-products of lactose catabolism, lactate, acetate, ethanol and CO₂, oxygen had no influ-

ence on lactate and CO₂ concentrations but only acted on acetate and ethanol production (Table 2). Acetate production increased progressively, at the expense of ethanol, so that the total molar concentration of acetate plus ethanol remained constant. The respiratory coefficient Q_r (CP/OU, mol CO₂/mol O₂) decreased progressively. A small production of acetate was observed in anaerobiosis with this strain.

Key role of ATP: the essential link between end-products and biomass production

According to the biochemical pathways of lactose, the ATP necessary for the building of new cells is provided as follows: one mole of ATP is produced by the breakdown of one mole of lactose, and 1 mole of ATP is produced for each mole of acetate synthesized (Condon 1987). The total ATP produced could therefore be calculated from experimental lactate and acetate concentrations and the instantaneous energetic efficiency Y_{ATP} could be determined (Y_{ATP} increase of biomass/ATP produced, g/mol) (Fig. 5).

Between 0 and 8 h, this value had no meaning because it corresponded to the lag phase and the beginning of the growth phase, lactate and acetate concentrations were too small to be determined with accuracy. After 8 h, Y_{ATP} had a fixed value

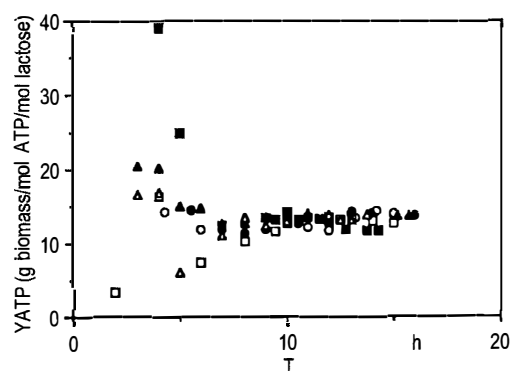


Fig. 5 Instantaneous energetic efficiency, Y_{ATP} : ○ anaerobiosis; ● air, 60 l/h; △ air, 120 l/h; ▲ air, 240 l/h; □ O₂, 36 l/h; ■ O₂, 120 l/h

Table 2 Comparison of end-products of lactose metabolism accumulating during aerobic and anaerobic cultures. Q_r is the respiratory coefficient

Aeration	Metabolic end products (mM)					
	D-Lactate	AcOH	EtOH	AcOH ethanol	CO ₂	Q_r (mol CO ₂ /mol O ₂)
Anaerobiosis	219	17	213	230	212	
Air, 60 l/h	214	88	153	241	201	2.86
Air, 120 l/h	224	107	122	229	215	2.14
Air, 240 l/h	217	122	105	227	198	1.68
O ₂ , 36 l/h	220	164	67	231	204	1.41
O ₂ , 120 l/h	226	227	6	233	204	1.11

Table 3 Times (*t*) when final biomass, lactose, lactate and acetate concentrations were reached for each aeration studied

Aeration	<i>t</i> _{biomass} (h)	<i>t</i> _{lactose} (h)	<i>t</i> _{lactate} (h)	<i>t</i> _{acetate} (h)
Anaerobiosis	15	15	15	9
Air, 60 l/h	14	14	14	14
Air, 120 l/h	14	14	14	14
Air, 240 l/h	14	14	14	14
O ₂ , 36 l/h	12.75	14	14	14
O ₂ , 120 l/h	12.75	14	14	14

of 14 whatever the aeration. This value was observed until the stationary phase was reached for batch cultures under pure oxygen and then fell to 11.5 because some acetate and lactate were produced after growth stopped (see Table 3). Some ATP was therefore produced without being used for cell building.

Discussion

The statement that many lactic acid bacteria are rather microaerophilic or anaerobic is not justified according to our results. Some strains do not effectively react in oxygen but they seem to be very rare (Johnson and McCleskey 1957). However, our data on growth under pure oxygen show that particular attention must be paid to aerobic growth. *L. mesenteroides* can vigorously consume oxygen when given the opportunity, and better growth yields and specific growth rates are obtained. Maximal biomass production in the presence of oxygen is difficult to achieve since the complete switch from ethanol to acetate only occurs under a 120-l/h flow rate of pure oxygen. It is interesting to see that maximal oxygen uptake and then maximal growth are not achieved with a 36-l/h flow rate of pure oxygen, although oxygen transfer is no longer limiting in this case. This result proves that oxygen consumption by our bacteria does not depend on limitation by oxygen, which is the reverse of what is generally admitted, but only on the oxygen transfer rate (OTR).

When working aerobically, the theoretical growth yield per mole of sugar ($Y_{X/S}$, g/mol lactose) is often stated as being as twice its anaerobic value (Condon 1987). This statement is only true when no more ethanol is produced, because ATP synthesis is doubled in this extreme case compared to anaerobiosis. Theoretical $Y_{X/S}$ values actually represent the maximal $Y_{X/S}$ that can be reached. Indeed, our results show that the phenomenon is more complex, since the simple fact of aeration does not completely eliminate the ethanol pathway. The switch from ethanol to acetate is not an on/off phenomenon and depends on oxygen consumption. In addition our results lead us to propose a simple method of detecting when maximal $Y_{X/S}$ is reached in aerobiosis: analysis of ethanol concentration. If no more ethanol is produced, no more growth is possible.

Also, in the case of acetate production in anaerobiosis, the maximal $Y_{X/S}$ is not doubled compared to the anaerobic value, as revealed by our data. This result agrees with previous work (Yashima et al. 1971; Lucey 1985) which shows that acetate production among *Leuconostoc* strains occurs in anaerobiosis. The small amounts of acetate produced in anaerobiosis do not come from citrate catabolism because, in our fermentative conditions (pH fixed at 6.5), citrate is only used in growth metabolism (Cogan et al. 1981).

Kinetic studies were carried out on variations in the maximal specific growth rate (μ_{\max}) versus OU_{\max} and suggested that a model similar to the Monod model should be applied, as follows:

$$\mu_{\max} = \mu_{0\max} + \mu_{\max}^* \cdot OU_{\max} / (K + OU_{\max})$$

where $\mu_{0\max}$ is the value of μ_{\max} in anaerobiosis, μ_{\max}^* is the absolute specific growth rate, and K is a constant. The data were plotted in Lineweaver-Burk plots ($1/\mu_{\max}$ versus $1/OU_{\max}$) yielding to the following values: $\mu_{\max}^* = 0.33 \text{ h}^{-1}$; $K = 67.6 \cdot 10^{-3} \text{ mol/l}$; $\mu_{0\max} = 0.45 \text{ h}^{-1}$, according to the experimental data.

Oxygen consumption in aerobiosis shows that the NADH oxidase pathways are open (Whittenbury 1974; Lucey 1985). Indeed, oxygen acts as an electron acceptor, which permits reoxidation of reduced pyrimidine nucleotides (Condon 1987). H_2O and H_2O_2 are end-products of NADH oxidase action; however, H_2O_2 is considered to be a toxic product for lactic acid bacteria (Piard and Desmazeaud 1991) and can inhibit growth when reaching high concentrations. However, Lucey (1985) showed that no inhibition of growth occurred in spite of H_2O_2 accumulation among seven strains of leuconostocs studied, although H_2O_2 concentrations did reach high levels (1 mM) for some strains. The abilities of lactic acid bacteria to react towards oxygen seem very variable, even if they belong to the same species. Whatever the NADH oxidase end-products, growth does not seem to be affected in our case.

Leuconostocs essentially use nitrogen constituents to build their new cells (Chauvet et al. 1982). That there is no growth without lactose confirms that lactose is a source of energy. Y_{ATP} remains constant for a batch culture whatever the aeration. Its high value (14 g/mol) compared to the 10 g/mol generally obtained (Doelle 1975; Salou et al. 1993) in anaerobiosis proves that aeration tends to act positively on ATP assimilation and proves that there is no waste during cell elaboration. A decrease of Y_{ATP} under pure oxygen seems to be attributable to a lack of amino acids or proteins, or salts and vitamins, since some lactose remains when growth stops. Indeed, inhibition by pure oxygen is not possible because no growth would occur as soon as the medium was sparged with pure oxygen. Our next aim is therefore to optimize the modified MRS medium under pure oxygen in order to keep Y_{ATP} constant until all the lactose is broken down, so achieving better growth yields.

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