Online-analytical characterization of 2,3-butanediol production by *Bacillus licheniformis* DSM 8785

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Abstract

Biodiesel is gaining increasing importance as an attractive fuel due to the depleting fossil fuel resources. Hence, it is important to develop processes with higher flexibility and quality to improve its production. Microbial production of 2,3-butanediol (2,3-BD) is a good example of that, with a history of more than 100 years. By developing an efficient bio-based process for the microbial production of 2,3-BD from renewable resources, fossil fuel supplies can be preserved and environmental benefits can be obtained.

Bacillus licheniformis conducts the metabolic pathway of 2,3-BD fermentation under microaerophilic conditions and is, thus, an effective producer of 2,3-BD. Oxygen is a limiting substrate with regard to growth and an inhibitor with regard to the specific metabolite productivity. The study of 2,3-BD production by *B. licheniformis* DSM 8785 was carried out during batch cultivations and bioreactor scale in consideration of medium composition (carbon source and concentration) and cultivation parameters (e.g. temperature, stirring speed) by applying different online analytical techniques to develop an improved fermentation process. Shake flasks in a Respiration Activity Monitoring System (RAMOS-device) were used to characterize the microbial respiration activity, oxygen transfer rate (OTR), and to study the metabolism and growth of the bacteria. The initial substrate concentration and temperature effected the yield of 2,3-BD production and the OTR profile. From 180 g/L of glucose at 30°C and 200 rpm, 26 g/L 2,3-BD were produced. The results from shake flask scale provided data to perform fermentations in a 3 L-bioreactor. Online-analyzes of DOT (Dissolved oxygen tension), redox potential, pH and stirred rate influence the 2,3-BD productivity. Oxygen transfer coefficients (k_La) of 58.65 h^{-1} and 53.72 h^{-1} were found to be optimal for conversion of 60 g/L and 180 g/L of glucose, respectively. 180 g/L of initial glucose concentration, 30°C, 400 rpm and an aeration rate of 0.5 vvm were found to be the optimal conditions for a successful scale-up by *B. licheniformis* DSM 8785.

Key words: *2,3-Butanediol; Oxygen limitation; online analysis; RAMOS device; Oxygen Transfer Rate (OTR); Bacillus licheniformis*

1. Introduction

1.1. 2,3-Butanediol

2,3-butanediol (2,3-BD) is a bulk-chemical with a large number of industrial applications. The heating value of 2,3-BD of 27 198 J/g is comparable to ethanol (29 055 J/g) and methanol (22 081 J/g). Thus, 2,3-BD or its derivatives might be easily used as a liquid fuel (Celinska, 2009).The production of 2,3-butanediol (hereafter referred to as 2,3-BD) assumed particular importance after the Second World War due to the need for synthetic rubber. By developing an efficient bio-based process for the microbial production of 2,3-BD from renewable resources, fossil fuel supplies can be preserved and environmental benefits can be obtained.

The IUPAC name is butane-2,3-diol and its molecular formula is $CH_3CH(OH)CH(OH)CH_3$. The molecular weight is 90.12 g/mol and in physical aspects, this compound is colorless, may be presented as an odorless liquid or in crystalline form, is hygroscopic and soluble in water (Syu, 2001). Due to the presence of two chiral carbon centers, the 2,3-BD has three stereoisomers.

Figure 1 the three isomers of 2,3-BD are depicted: the optically inactive isomer (*R*,*S*)-2,3-BD, also known as meso-2,3-BD; the optically active forms are (*2R,3R*)-BD or D-(-)-2,3-BD and (*2S,3S*)-BD or L- (+)-2,3-BD.

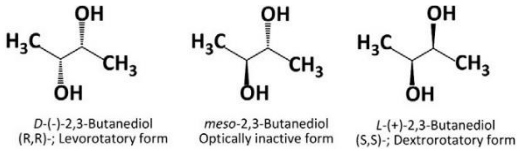

Figure 1 *Stereoisomers of 2,3-BD (Ji X.-J. , 2011)*

The production of 2,3-BD is of great interest because of the various applications of 2,3-BD. It can be used in the manufacture of printing inks, perfumes, chemicals, foods, fumigants, moistening agents, fuels, explosives, plasticizers, pharmaceuticals and aeronautical products. 2,3-BD can be derived from the bioconversion of natural resources (Ji X.-J. e., 2011; Lan Ge, 2011). 2,3-BD production is growing at an annual rate of 4-7%, Since 2,3-BD has a unique structure and its chemical synthesis is pricey, large scale chemical synthesis has not been established. Considering the limitations of the chemical industry to produce pure 2,3-BD most of the 2,3-BD is not sold as a separate product, but mixed with other products (Lan Ge, 2011; Syu, 2001).

1.2. Microbial 2,3-BD producers and Metabolic pathway

Microbial 2,3-BD production dates back to 1906, when Harden and Walpole reported for the first time results about the 2,3-BD synthesis with *Klebsiella pneumoniae.* Industrial-scale production of 2,3-BD by fermentation was first proposed in 1933 by Fulmer EI et al (1933).

2,3-BD fermentation regained interest in the last decade mostly because the fermenting sugars and lignocellulosic hydrolysates are cheap substrates. Since that, numerous efforts have been made in improving fermentation processes. Many bacterial strains are able to synthesize 2,3-BD from pyruvate. However, only a few are able to produce 2,3-BD in significant quantities.

Bacterial species considered to be of industrial importance in 2,3-BD production belong to the genera *Klebsiella, Enterobacter, Bacillus* and *Serratia* (Ji X.-J. , 2011). Some investigations were carried using native producers, such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens* and *Enterobacter aerogenes* (Jiang Y. e., 2014). Since these microorganisms belong to risk group 2 (pathogenic) they are not favorable for industrial-scale fermentation and 2,3-BD producers belonging to group 1 (safe) would be highly preferred, as *Bacillus* species. The purpose was to find the non-pathogenic microorganisms for the production of 2,3-BD at developed bioprocesses in large scale, which is possibly less expensive.

During bacterial metabolism, monosaccharides or other carbon sources can be converted to 2,3 - BD via pyruvate as a central metabolic intermediate. Pyruvate is the branch point where the catabolic reactions diverge into two different energy-production pathways. Either pyruvate is channeled via acetyl-CoA into the tricarboxylic acid cycle under aerobic conditions or it is subject to the mixed-acid-2,3-BD fermentation under anaerobic conditions in three steps. In the branch leading to 2,3-BD, the first step is the decarboxylation of pyruvate to α-acetolactate in the presence of the enzyme α-acetolactate synthase. In the second, α-acetolactate is converted into acetoin (acetyl methyl carbinol) by the enzyme α-acetolactate decarboxylase. In a third step, acetoin is reversibly reduced to 2,3-BD by the enzyme acetoin reductase (2,3-BD dehydrogenase) (Ji X.-J. , 2011). The reversible reaction between acetoin and 2,3-BD can influence the maintenance of the intracellular $NADH/NAD^+$ balance (Blomqvist, 1993).

1.3. Factors affecting the 2,3-BD production

Many cultural, environment and nutritional factors can affect the 2,3-BD fermentation due to the metabolism of 2,3-BD. In this chapter some pH,

temperature, aeration, agitation, medium and substracte composition will be introduced.

In general, the pH is a fundamental parameter in the regulation of bacterial metabolism. The influence is especially important in the processes involving multiple end-product formation like in mixed acid 2,3-BD fermentation. The pH of the fermentation medium affects the biomass composition and the metabolic pathway that the bacteria take.

The efficiencies of bioprocesses are strictly temperature-dependent due to the strong dependence of enzymatic activity and cellular maintenance upon temperature. Since 2,3-BD synthesis is a growth-associated phenomenon, the optimum temperature for product formation should be similar to the optimum temperature for maximum biomass yield (Garg & Jain, 1995).

One of the most important parameter for 2,3-BD production is considered to be the oxygen availability. 2,3-BD production is a product of anaerobic fermentation and is formed under oxygen limited or microaerophilic conditions. Nevertheless, aeration was shown to enhance 2,3-BD synthesis, particularly at high substrate concentrations or during the fermentation of pentose sugars (Celinska, 2009; A. Singh, 1995). Agitation has not only a strong impact on mixing but also on the oxygen supply in fermentation processes. Therefore, agitation has likewise an effect on 2,3-BD fermentation (Celinska, 2009).

A culture medium must contain all the essential nutrients that the particular microorganism needs for growth and maintenance. Nevertheless, for an efficient 2,3-BD synthesis some additives like vitamins and trace elements have to be supplemented (Garg & Jain, 1995; Ji X.-J., 2011). 2,3-BD yield and production rate often depended on the particular raw material used and the initial sugar concentration. Some studies suggested that when the sugar concentration in the raw material is increased, the level of toxicity also increases resulting in poor substrate utilization (Jansen N. B., 2005). Therefore, in industrial-scale fermentations substrates are frequently diluted to lower sugar concentrations (Voloch M, 1985).

1.4. Scale-up process

Anaerobic fermentations take place in the absence of oxygen; in these processes, multiple compounds can act as oxidizing agents, such as sulfate or nitrate. Thus, it is important to ensure an adequate delivery of oxygen from a gas stream to the culture broth. As consequence, a precise estimation of the oxygen transfer rate (OTR) at different scales and different operational conditions has a relevant role for the prediction of the metabolic pathway and a crucial importance for the selection, design and scale-up of bioreactors. The mass transfer rate may be influenced by the chemical rate of the bioprocess. For instance, oxygen is consumed by the suspended microorganism, and therefore an enhancement of the OTR takes place. The OTR is one of the most important factors in 2,3-BD fermentation (Suresh S, 2009).

The determination of volumetric oxygen mass transfer coefficient (k_La) in bioreactors is essential in order to establish aeration efficiency and to quantify the effects of the operating variables on the provision of dissolved oxygen. The measuring of the respiratory activity, or respirometry, is a powerful tool for monitoring and controlling industrial fermentation processes. The ratio of the net molar quantity of CO_2 evolved by a microorganism (CTR) and the molar quantity of oxygen accordingly consumed (OTR) is called the Respiratory Quotient (RQ). The RQ provides precious information about the state of a culture, such as the substrate on which the microbe is growing.

The effects of reactor operation mode on 2,3-BD formation is important to establish an optimal process design. Different operation modes were tested including batch, fed-batch, continuous culture, cell recycle and immobilized cell systems. In order to recover 2,3-BD from the fermentation broth product concentrations above 80 g/L are required (Ji X.-J. e., 2011).

2. Materials and Methods

2.1. Microorganism

The microbial strain *Bacillus licheniformis* DSM 8785 employed in this thesis was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Bacterial cultures (Braunschweig, Germany).

2.2. Medium composition and culture conditions

All the chemicals used in the conducted experiments were purchased from one of the following companies: Sigma-Aldrich Chemie GmbH (Steinheim, Germany), Carl Roth GmbH & Co. KG (Karlsruhe, Germany), VRW International GmbH (Kelsterbach, Germany) and Merck KGaA (Darmstadt, Germany).

For cultivation, the basal medium described by Nakashimada et al. (1998) was used. The medium contained (per liter): 5 g yeast extract, 5 g tryptone, 7 g K_2HPO_4, 5.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4.7H_2O$, 0.12 g $Na_2MoO_4.2H_2O$, 0.021 g $CaCl_2.2H_2O$, 0.029 g $Co(NO_3)_2.6H_2O$, 0.039 g $(NH_4)_2Fe(SO_4)_2.6H_2O$ and 10 ml trace elements solution contained 0.2 g nicotinic acid, 0.0262 g $Na_2SeO_3.5H_2O$, 0.0037 g $NiCl_2.2H_2O$, 0.5 g $MnCl_2.4H_2O$, 0.1 g H_3BO_3, 0.0172 g $AlK(SO_4)_2.12H_2O$, 0.001 g $CuCl_2.2H_2O$ and 0,554 g $Na_2EDTA.2H_2O$ per liter.

Tbe medium components were prepared as separate solutions and mixed together after autoclaving (21 min, 121 ºC). Trace elements and the solution containing $Co(NO_3)_2.6H_2O$ and $(NH_4)_2Fe(SO_4)_2.6H_2O$ were sterile-filtered into the medium. Separately autoclaved glucose was supplemented to the desired initial concentration.

2.3. Shake flasks in RAMOS and 3L-bioreactor scale

All inocula were prepared using RAMOS flasks. 25 mL culture medium (containing the initial glucose concentration for each experiment) was incubated with 1 mL of cells from a Master Bank Cells prepared before. Precultures were carried out at 30°C and 200 rpm until the exponential phase was reached.

Cultivation experiments in the RAMOS device were carried out at different filling volumes to adjust different maximal oxygen transfer capacities (Anderlei T. a., 2001). Each filling volume was conducted in duplicate. The initial optical density (600 nm) was adjusted to 0.4. Initial medium pH ranged from 6.4 to 6.7. The shaking frequency was 200 rpm and the shaking diameter was 5 cm.

For the fermentation in a 3 L-Applikon Biotechnology (Schiedam, The Netherlands) fermenter, bioreactor cultivations with a working volume of 2L were performed. Yeast extract, tryptone, K_2HPO_4 and KH_2PO_4 were filled into the bioreactor and autoclaved together, while glucose containing the sugar source was autoclaved separately and added aseptically afterwards. The other medium components were injected into the bioreactor via a septum. The temperature was controlled at 30 ºC and the stirrer speed set to 400 rpm. During the first 3 h of the first cultivation the stirrer speed was varied to characterize the bioreactor with respect to the oxygen mass transfer coefficient (k_La-value). The aeration rate was 0.5 vvm.

2.4. Analytical methods

Samples of the RAMOS experiments were only taken in the end of the main cultivation and samples of the bioreactor cultivations were taken every day.

2.4.1. Optical density

The OD measurements were performed at 600 nm using a *Genesys 20* spectrophotometer (Thermo Scientific, USA). Samples were diluted with Basal medium so that the absorbance was in the linear range of the photometer between 0.1 and 0.6.

2.4.2. Cell Dry Weight (CDW)

For the bioreactor samples the cell dry weight was determined. 2 mL of culture broth were centrifuged for 10 min at 13.000 rpm in pre-

weighed centrifuge tubes using a Sigma I-15 centrifuge with a plastic rotor include hermetic plastic (Sigma Laborzentrifugen GmbH, Germany). The supernatant was discarded, the cell pellet dried at 60°C over night and weighed afterwards.

2.4.3. pH measurement

Offline pH measurements of media or culture samples were performed using a digital pH-meter (OAKTON PH 510, Netherlands). In order to ensure the accuracy of the pH electrode, a two-point calibration with buffer solutions of pH 4 and pH 7 was conducted on a daily basis.

2.4.4. HPLC analysis

Glucose, 2,3-BD and by-products were detected by high performance liquid chromatography (HPLC) using the organic-acid-resin (250x8 mm: CS-Chromatographie GmbH, Langerwehe, Germany). The column was operated at 85 °C, and 0.005 M H_2SO_4 with a flow rate of 0.8 ml/min was used as mobile phase. The column allows a good differentiation of *meso-2,3-BD* from the D-(-)-2,3-BD and L-(+)-stereoisomers of 2,3-BD.
Before HPLC measurement, samples were centrifuged for 10 min at 13.000 rpm. The supernatant filtered through a polyester filter (pore size 0.20 µm; Macherey-Nagel GmbH & Co. KG, Düren, Germany) and filled into HPLC sample vials.

3. Results

3.1. 2,3-BD production in special shake flasks

Investigations regarding the optimization of glucose concentration and of cultivation parameters were conducted on a shake flask scale. An experiment with the optimal parameters reported by Jurchescu et al. (2013) was performed. This study took into consideration the optimum initial glucose concentration for high 2,3-BD production. The incubation temperature and the OTRmax were also investigated.

Seven different shake flasks, with different numbers and shapes of baffles, were used to carry out batch cultivations with an initial concentration of 180 g/L of glucose. The aim of this experiment was to study the effect of baffles in shake flasks on the oxygen transfer, as well as 2,3-BD production by *B. licheniformis* DSM 8785 and to transfer the conditions from Jurchescu's study, non-baffled shake flasks. Figure 2 represents the different configuration between baffled shake flask and non-baffled shake flask.

Figure 2 Different shape configuration of shake flasks with and without baffles from the Biochemical Engineering at the RWTH Aachen University

Baffles have been used in shake flasks to increase oxygen transfer, as well as to prevent vortex formation and make the culture medium more homogenous.
Figure 3 presents the results for oxygen transfer rate, carbon dioxide transfer rate and respiratory quotient for different shake flasks and the same medium conditions that Jurchescu (2013) used. OTR and CTR are important to characterize a culture and provide more detailed process knowledge.
All the cultures in shake flasks presented a short lag phase and a faster exponential phase. However, each culture reached different plateau, which depend the format and the number of baffles of the shake flask.
The two medium cultures in standard shake flasks (profile with square in black and white) showed the lowest value for CTR and OTR, but with a longer plateau. This happened because there were no baffles in the flasks. One of the flasks with two medium baffles (profile with diamond filled with blue), a diauxic growth can be

observed from the CTR and OTR profiles, indicating a change in substrate.

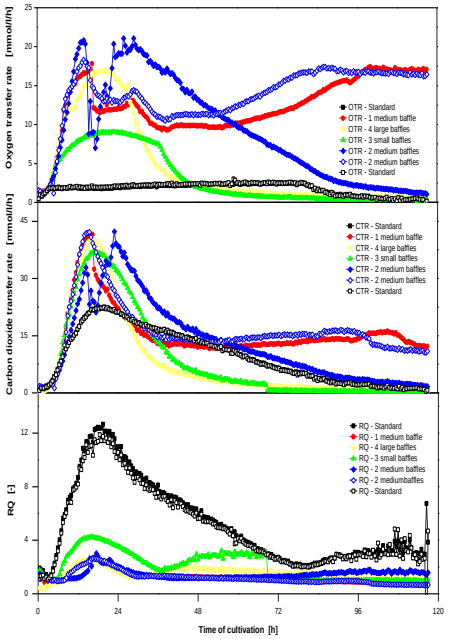

Figure 3 **Effect of flask configuration in OTR, CTR and RQ on 2,3-BD production with *B. licheniformis* DSM 8785**. Conditions: 500 mL shake flasks, 100 mL medium containing 180 g/L glucose, T=30 °C, N=100 rpm, d0= 5 cm and initial pH 6.5

When the primary carbon source is exhausted, the cell starts to consume acetate if no other more readily metabolized substrates are available. (Celinska, 2009). On other hand, the shake flask with two medium baffles and the flask with one medium baffle obtained the maximum OTR and CTR, close to 16 mmol/l/h. The oxygen limitation was achieved after 96 hours of fermentation. It can be also observed that with standard flasks the RQ value is higher than the one obtained with flasks with baffles, being the ratio close to 12 after 20 h of fermentation. The cultures in flasks with baffles show a ratio lower then 3, nevertheless the flask with three small baffles stands out, with a ratio of 4 after approximately 16 hours of fermentation. In the end of the experiment, for each flask, a sample of main cultivation was analyzed by HPLC. The results can be shown in Figure 4.

All cultures in shake flasks with baffles produced ethanol, while cells in standard flasks did not produce it. At an extremely low oxygen level, big amounts of anaerobic fermentation products can

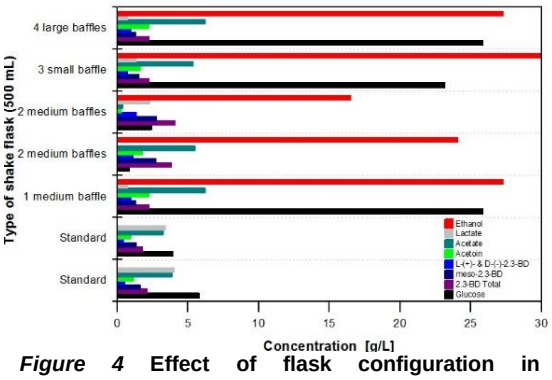

Figure 4 **Effect of flask configuration in fermentation end-products and 2,3-BD production with *B. licheniformis* DSM 8785**. Conditions: 500 mL shake flasks, 100 mL medium containing 180 g/L glucose, T=30 °C, N=100 rpm, d0= 5 cm and initial pH 6.5.

be formed. Oxygen supply is better in baffled flasks, so the HPLC results should show a higher 2,3-BD production and aerobic fermentation products. However, in all the shake flasks, the cultures had remained with glucose. The shake flasks with two medium baffles, with three small baffles and with four large baffles showed higher concentrations of glucose. 2,3-BD production was higher in the shake flasks with 2 baffles and 4.1 g/L of 2,3-BD was produced.

3.2. 2,3-BD production with RAMOS flask experiments

Batch cultivations in shake flasks were carried out using various initial glucose concentrations for 2,3-BD production with *B. licheniformis* DSM 8785 demonstrates a comparison between the influence of the medium volume with 180 g/L or 50 g/L initial glucose concentration at 30 ºC on OTR, CTR and RQ, as Figure shows.

In the experiment with 50 g/L of initial glucose concentration, the OTR and CTR of eight shake flasks exponentially increased until about 12 h where they leveled off on plateaus of 13 mmol/l/h and 16 mmol/l/h; 12 mmol/l/h and 10 mmol/l/h; 14 mmol/l/h and 14 mmol/l/h; 12 mmol/l/h and 10 mmol/l/h for filling volumes of 10 mL, 15 mL, 20 mL and 25 mL, respectively.

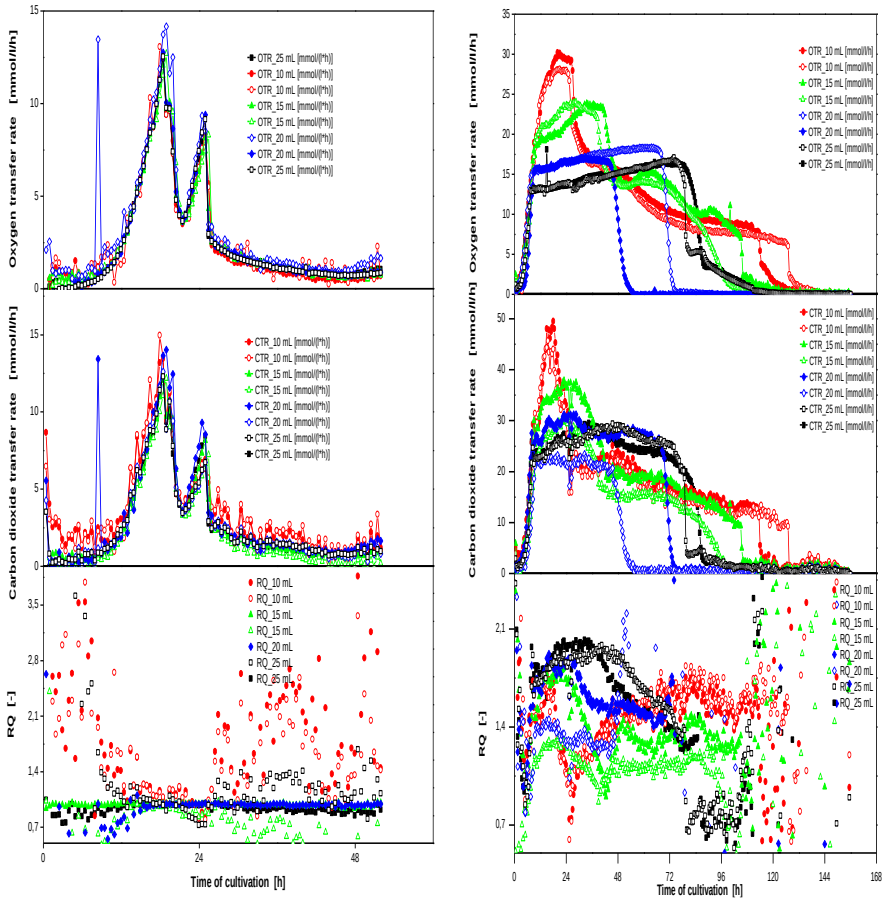

Figure 5 **Comparison of OTR, CTR and RQ during cultivation with *B. licheniformis* DSM 8785 using different initial glucose concentrations and filling volumes**. Conditions: two shake flasks with 10, 15, 20 and 25 mL with medium containing 50g/L - pH=6.60 (left side) and 180g/L - pH=6.69 (right side) of initial glucose concentration, T=30 °C, N=200 rpm and d0= 5 cm

After ca. 18 h the oxygen and carbon dioxide transfer rates heavily dropped down, because the essential substrate, glucose, was completely exhausted. However, a second peak was verified between 20 and 28 h by the oxygen transfer rate and carbon dioxide transfer rate at lower transfer rates of 8 mmol/l/h. With an initial glucose concentration of 180 g/L the OTR and CTR exponentially increased in parallel during the first 10 hours. The oxygen transfer rates again leveled off on maximum plateaus of approximately 30 mmol/l/h, 24 mmol/l/h, 16 mmol/l/h and 15 mmol/l/h with 10 mL, 15 mL, 20 mL and 25 mL of filling volume, respectively. For carbon dioxide transfer rate with 10 mL, 15 mL, 20 mL and 25 mL of filling volume a plateau of approximately 50 mmol/l/h, 40 mmol/l/h, 38 mmol/l/h and 28 mmol/l/h was achieved, respectively. The oxygen limitation lasted longer with 180 g/L than with 50 g/L glucose because the bacteria needed more time to consume the larger amount of glucose. The double growth, with 50 g/L of glucose, is known as diauxic growth. Diauxic growth is a double growth and can be caused by the presence of a second carbon source. In this case, the glucose, the preferred sugar, was consumed first, which led to rapid growth and afterwards, the offline analyses (not shown) succinate and ethanol depletion. The bacterial culture with 10 mL of filling volume shows a RQ value, for the most part, above 1 with the experiment with 50 g/L of glucose. With 180 g/L glucose, all the bacterial cultures show a RQ value between 0.8 and 2.These values corresponding to the acetate and succinate consumption.

3.3. Scale-up of 2,3-BD with *Bacillus licheniformis* DSM 8785 to 3L-bioreactor scale

In bioprocesses the oxygen availability to the microbial cell strongly affects growth and product formation. Therefore, the oxygen transfer rate as well as the dissolved oxygen tension (DOT) were investigated in the bioreactor experiments. Figure shows the OTR, DOT, and derived potential redox as well as the CTR and RQ.

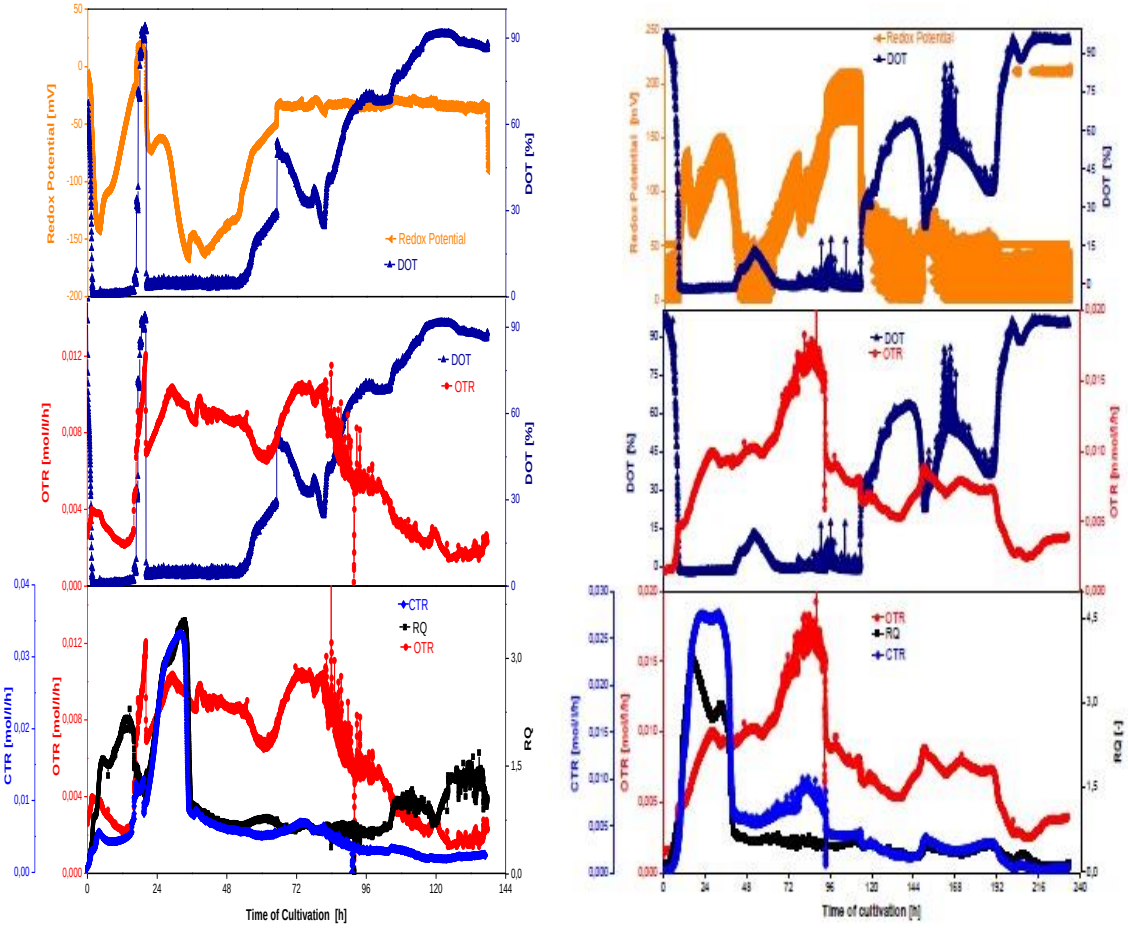

Figure 6 Influence of the dissolved oxygen tension in the profile of potential redox, OTR, CTR and RQ. Batch fermentation of 2,3-BD from 60 g/L /left side) and 180 g/L (right side) of initial glucose concentration by *B. licheniformis* DSM 8785. Fermentation was performed at 30 ºC in a 3-L bioreactor with stirring at 400 rpm and airflow at 0.5 vvm.

From the depicted with 180 g/L, illustrates the influence of redox potential and dissolved oxygen tension in the media with 180 g/L of initial glucose concentration by *B. licheniformis* DSM 8785. The fermentation started with DOT decreased, while redox potential increased faster in the first 10 h of fermentation. Redox potential dropped until hour 12 and DOT remained constant. This indicated an oxygen limitation of the microbial culture. After, redox potential rose until 150 mV between hour 18 and 40. After hour 36, DOT increased and redox potential decreased, glucose has been consumed. Consequently, the DOT decreased and redox potential increased until hour 88. After approximately 112 h the acetate is depleted, DOT increased and potential redox decreased and two plateaus at approximately 60% until hour 150 and 192, respectively. The profile of DOT is a stronger indicator for a diauxic change in microbial growth behavior.

The OTR exponentially increased after a short lag-phase, while DOT value dropped fast until 10 h after the beginning of fermentation. After 28 h, the oxygen transfer rate levels off and plateaus at approximately 10 mmol/l/h until hour 60, indicating an oxygen limitation. After that, the OTR continued to increase until 80 h where achieved the maximum oxygen limitation close to

20 mmol/l/h. The plateau of the oxygen transfer rate between 72 and 96 h represented the maximum oxygen transfer rate capacity of the system, the fermentation of the bacteria was oxygen-limited in this phase. The subsequent dropped down of the oxygen transfer rate indicated the depletion of the carbon source.

The carbon dioxide transfer rate and respiratory quotient raised exponentially during the first 10 h. CTR achieved 28 mmol/l/h and RQ a ratio of 4. After 36 h, the CTR have been a sharp decreased what indicated that the glucose has been consumed. In this phase, the respiratory quotient had decreased until hour 30 and after an exponential growth until a RQ value of 3. After 60 h, a softly increase began in the CTR. During this phase, the bacterium used the respiro-fermentative glucose metabolism and produced anaerobic products, as the OTR profile shows. After 84 h the carbon dioxide transfer rate decreased first fast and after steeply, which agrees with the exhaustion of the initial carbon source glucose. The RQ was around 0.7 in this phase and decrease afterwards close to 0.

For 60 g/L, online measurement of fermentation, as potential redox, have an important role in the physical state of fermentation media and in the oxygen system. DOT and redox potential dropped until hour 12. The DOT curve shows a peak which indicated a short interruption of metabolic activity and the growth. The measured values for DOT can indicated the switching of the carbon sources between 12 and 20 h or the changed in the stirred when the bioreactor was set up to 400 rpm. Its amplitude further increased after 55 h, what normally referred to an increase of biomass. Afterwards, DOT and redox potential raised until the maximum value of 20 mV and 90%,

The OTR curve, the first growth phase on glucose lasted until 12 h. Nonetheless, the DOT curve, which returns at the same time nearly to its initial value, together with the OTR curve. After 24 h, the profile of DOT and OTR were a different behavior. OTR increased until achieved a plateau, 0.010 mol/l/h. The plateau of the oxygen transfer rate between 30 hours and 60 represented the maximum oxygen transfer rate capacity of the system. This implied that the fermentation of the bacteria is oxygen-limited in this phase. The drop in the oxygen transfer rate at hour 60 indicated that the carbon source had been completely exhausted and a DOT value increase to 60%. Then, between 60 and 72 h, a new plateau was reached. The reason for the observed increasing may be due to secondary metabolites were consumed. The plateau of the oxygen transfer rate was between 72 hours and 86 approximately. Subsequently, a constant decrease in the OTR-value happened and DOT value increased until 90%, what meant that the fermentation was coming to an end.

The carbon dioxide transfer rate rose during the first 24 h, 30 mmol/l/h. After 36 h, the CTR has been a sharp decrease, what indicated that the glucose has been depleted. In this phase, the respiratory quotient had an exponential growth, as can be observed for the maximum 3.5 RQ value. After 60 h, a softly increase began in the CTR. After, 72 hours the carbon dioxide transfer rate decreased steeply, which agrees with the exhaustion of the initial carbon source glucose. The RQ was around 0.7 in this phase, indicating the consumption of a carbon source, ethanol, which is more reduced than glucose.

4. Discussion

The cultivation experiments conducted in special shake flasks with baffles attempted to replicate the results from Jurchescu et al. (2013). The results showed that the highest level of 2,3-BD (4.1 g/L) was obtained on medium with 180 g/L of initial glucose, 30 ºC and in the flask configuration with 2 baffles and 1 baffle. An increase in the number of baffles resulted in a corresponding lower production of 2,3-BD. The theoretical yield of 2,3-BD production from glucose is 0.5 g/g used sugar (Voloch M, 1985) The highest productivity obtained in the present study was 0.056 g/(L*h), reached on medium after 40h. It was thus not possible to reproduce the results from Jurchescu

et al (2013), since 0.42 g/g glucose is much higher then 0.023 g/g.

Batch experiments performed with *B. licheniformis* DSM 8785 indicated that 2,3-BD production is influenced by the amount of glucose added to the culture medium. Starting with an amount of glucose of 50 g/L, an increase of the initial glucose concentration (up to 180 g/L) resulted in a higher production of 2,3-BD. A maximum of 26 g/L 2,3-BD was reached from 180 g/L glucose. The yield (g/g glucose) was 0.15 and the volumetric productivity 0.87 g/(L*h). Experiments with 50 g/L were carried out to check the osmotic pressure inside the shake flasks. Measurement of osmotic pressure is an important tool in the development of the experiment characterization. The maximum oxygen transfer rate (OTR) for all the experiments was analysed with different filling volumes. The lower the volume of medium, the better was the OTR and hence better results were attained.

From 250 mL of shake flasks in RAMOS, two fermentations in a bioreactor with 3L were carried out. The first experiment in the 3 L bioreactor tested seven different stirring speeds between 200 and 800 rpm. A stirring speed of 400 rpm was found optimal for bacterial growth and 2,3-BD production. Regarding online analysis, an average k_La of 58.65 h^{-1} and 53.72 h^{-1} were found optimal for 60 g/L and 180 g/L of initial glucose concentration, respectively. Relatively to the optimization of the temperature, 30 ºC was found to be best value for 2,3-BD production in the 3L bioreactor. The aeration rate was 0.5 vvm. Maximum 2,3-BD concentrations reached only 2.53 g/L on 60 g/L, while on 180 g/L medium 3.17 g/L 2,3- BD were obtained.

5. Conclusion and prospects

Shake flask with baffles cultivations showed that a glucose concentration of 180 g/L, 30°C and 100 rpm are optimal conditions for high 2,3-BD production with *B. licheniformis* DSM 8785. A maximum of 4.1 g/L 2,3-BD were obtained in batch experiments. Moreover, using the batch cultivation mode, with the RAMOS device, 2,3-BD production could be increased up to 26 g/L and the productivity up to 0.87 g/(L*h). Biotechnological production of 2,3-BD on an industrial-scale is still in its early stage, but with strong prospects of growth.

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