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# Influence of pH and aeration on 2,3-butanediol production from glucose by *Klebsiella pneumoniae* G31

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The strain *Klebsiella pneumoniae* G31, an extra-producer of 2,3-butanediol (2,3-BD) from glycerol, was tested in glucose fermentation. Key factors affecting the fermentation, such as pH and aeration regime were optimized. The best conditions favorable for 2,3-BD production were found to be pH 6.0, air flow 1.45 vvm and agitation speed of 500 rpm. Thereby, after 18 h of fermentation, the concentration of 2,3-BD reached a maximum of 30.4 g/l with productivity of 1.69 g/l h and yield of 0.32 g/g. The efficiency of the process demonstrates the potential industrial application of *K. pneumoniae* G31 as a 2,3-BD producer from glucose-containing feedstock.

**Keywords:** 2,3-butanediol, glucose, *Klebsiella pneumoniae*, pH, aeration.

## INTRODUCTION

The growing interest in 2,3-BD production in recent years is due to the wide range of industrial applications of diols. 2,3-BD and its derivatives as 1,3-butadiene, ethylketone and many others, are extensively used in many industrial sectors: manufacturing of printing ink, perfumes, explosives, softening agents, plasticizers, foods and pharmaceuticals [1,2,3]. In addition, its high octane rating makes 2,3-BD a potential aviation fuel [4].

2,3-butanediol can be produced *via* the mixed acid-alcohol fermentation pathway from carbohydrates. In certain conditions 2,3-BD is the major product, but accumulation of acids, ethanol and carbon dioxide cannot be avoided. The most preferable carbon source is glucose, although 2,3-BD can be produced naturally from different hexoses, pentoses, disaccharides or glycerol [5,6]. As the final price of the product strongly depends on the raw material cost, recently the focus is on the use of cheaper and abundant raw materials as molasses, whey, Jerusalem artichoke, crude glycerol, lignocellulosic materials, food industry residues or wood hydrolysates [2,7,8].

Although many species can generate 2,3-BD, only the strains of *Klebsiella pneumoniae* and *Klebsiella oxytoca* are capable of producing it in quantities high enough for industrial application. Of significant importance for the process efficiency are the fermentation conditions. The key role to enhance 2,3-BD yield and productivity, as well as to decrease by-product formation is played by the medium composition and pH and the oxygen supply. Since 2,3-BD is produced *via* mixed acid fermentation, usually the higher the pH of the media, the greater are the amounts of acids produced. On the other hand, lower values of pH lead to lower biomass formation and consumption rate. Hence, the highest 2,3-BD concentration, productivity and conversion rate could be obtained at different pH conditions. The oxygen supply also has a contradictory effect on 2,3-BD production. Jansen *et al*. [9] found that higher aeration rates increase biomass formation, but decrease 2,3-BD accumulation. On the opposite, lower oxygen supply decreases both conversion rate and cell density. Thus, aiming at shifting the metabolic flux toward 2,3-BD, a lot of new strategies of oxygen supply control [10], pH control [11], and medium optimization [12] were developed. In any case, the optimum values of the air flow, pH and agitation speed control are strain specific and should be experimentally determined.

## MATERIALS AND METHODS.

Strain. For the present experimental work the strain *Klebsiella pneumoniae* G31 was used, an extra-producer of 2,3-BD from glycerol [11,13], isolated from active slime [14] and deposited in the

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Bulgarian National Collection for Microorganisms and Cell Cultures under registration № 8650.

Media and cultivation conditions. The strain used for seed preparation was preserved at a temperature of -20ºC with addition of 20% glycerol. The fermentation medium used for both inoculums and batch processes was previously optimized for 2,3-BD production from glucose [15]. Content (g/l): $(NH_4)_2HPO_4$, 4.91; Na-acetate, 3; KCl, 0.4; $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.02; $MnSO_4.7H_2O$, 0.01; yeast extract, 5; $ZnSO_4.7H_2O$, 0.001. Inoculum cultures were supplemented with 30 g/l glucose, as 100 ml media were grown on a rotary shaker in 500 ml flasks at 37ºC, agitation 200 rpm for 24 h. The batch fermentations were performed in a 1 l stirred bioreactor (Biostat® A plus, Sartorius Stedim Biotech, Goettingen, Germany). The media for all batch experiments were supplemented with 100 g/l glucose and sterilized at 105 ºC for 15 min. Temperature was set to 37 ºC and 1% (v/v) of inoculum was added. The aeration regime and pH of the medium were the subjects of optimization. The air flow and agitation speed control were varied between 0 and 1.6 l/min (up to 1.6 vvm) and 200 – 700 rpm, respectively. The pH was adjusted by 5 M NaOH.

Analytical methods. Glucose, 2,3-butanediol, ethanol, acetic, lactic and succinic acid concentrations were determined using high-performance liquid chromatography (HPLC). The metabolites were separated using a Bio-Rad column for organic acids analysis (Aminex Ion Exclusion HPX-87H) at 65ºC and detected by RI detector (Perkin-Elmer series 10 HPLC). As a mobile phase 0.005 M sulphuric acid at an elution rate of 0.6 ml/min was used. Cell growth was estimated by measurement of the optical density of the broth at 600 nm ($OD_{600}$) with a UV-1600PC spectrophotometer (VWR).

## RESULTS

### *pH optimization.*

The pH was optimized in batch processes under microaerobic conditions (1.0 l/min sterile air supply and agitation at 200 rpm). The pH was maintained at 5.5, 6.0, 7.0 or not controlled. The results showed that the higher pH maintained, the greater was the extent of biomass formation and the conversion rate. At pH 7.0 after 18 h of fermentation, almost the entire amount of 100 g/l glucose was consumed, but the main product was lactic acid (21.04 g/l). In all other cases the main product was 2,3-BD, and the lower the pH of the medium, the higher was the conversion of glucose to 2,3-BD. Nevertheless, the highest 2,3-BD concentration (22.72 g/l) was obtained at pH 6.0, because at lower pH values the glucose consumption sharply decreased. Thus, when pH was not controlled (pH decreased to 4.5), the highest conversion of glucose to 2,3-BD was obtained (0.39 g 2,3-BD / g glucose consumed), but the maximum concentration was only 16.11 g/l (Table 1).

### *Aeration regime optimization.*

Batch fermentations with different air flow (1.0 - 1.6 vvm) and agitation speed (200 – 700 rpm) were investigated at non-controlled pH conditions. The results are given in Table 2.

**Table 1.** Glucose consumption and products formation after 18 h of fermentation in batch processes with different pH control. Air flow 1.00 vvm, agitation 200 rpm.

| Conditions | Glucose consumed (g/l) | 2,3-BD (g/l) | Yield of 2,3-BD (g/g) | Acetic acid (g/l) | Lactic acid (g/l) | Ethanol (g/l) | Succinic acid (g/l) | Biomass ($OD_{600}$) |
|---|---|---|---|---|---|---|---|---|
| uncontrolled pH | 40.82 | 16.11 | 0.39 | 1.67 | 2.01 | 3.44 | 1.37 | 2.013 |
| pH=5.5 | 62.81 | 19.20 | 0.31 | - | 7.08 | 4.65 | 3.54 | 2.150 |
| pH=6.0 | 73.77 | 22.72 | 0.31 | 0.49 | 8.27 | 5.63 | 3.13 | 2.353 |
| pH=7.0 | 97.06 | 19.21 | 0.20 | 4.84 | 21.04 | 8.62 | 3.85 | 2.678 |

**Table 2.** Glucose consumption and products formation after 18 h of fermentation in batch processes with different aeration. All fermentations were conducted without pH control.

| Aeration | | Glucose consumed (g/l) | 2,3-butanediol (g/l) | Acetic acid (g/l) | Lactic acid (g/l) | Ethanol (g/l) | Succinic acid (g/l) | Biomass ($OD_{600}$) |
|---|---|---|---|---|---|---|---|---|
| Air flow (vvm) | Agitation (rpm) | | | | | | | |
| 1.0 | 200 | 40.82 | 16.11 | 1.67 | 2.01 | 3.44 | 1.37 | 2.013 |
| 1.15 | 500 | 50.97 | 21.80 | 1.80 | 0.39 | 2.62 | 1.14 | 2.150 |
| 1.45 | 500 | 65.00 | 24.15 | 1.70 | 0.15 | 2.30 | 1.05 | 2.425 |
| 1.60 | 500 | 60.78 | 23.46 | 1.47 | - | 2.19 | 0.90 | 2.637 |
| 1.60 | 700 | 5.1 | 2.08 | 1.50 | - | - | - | 2.986 |

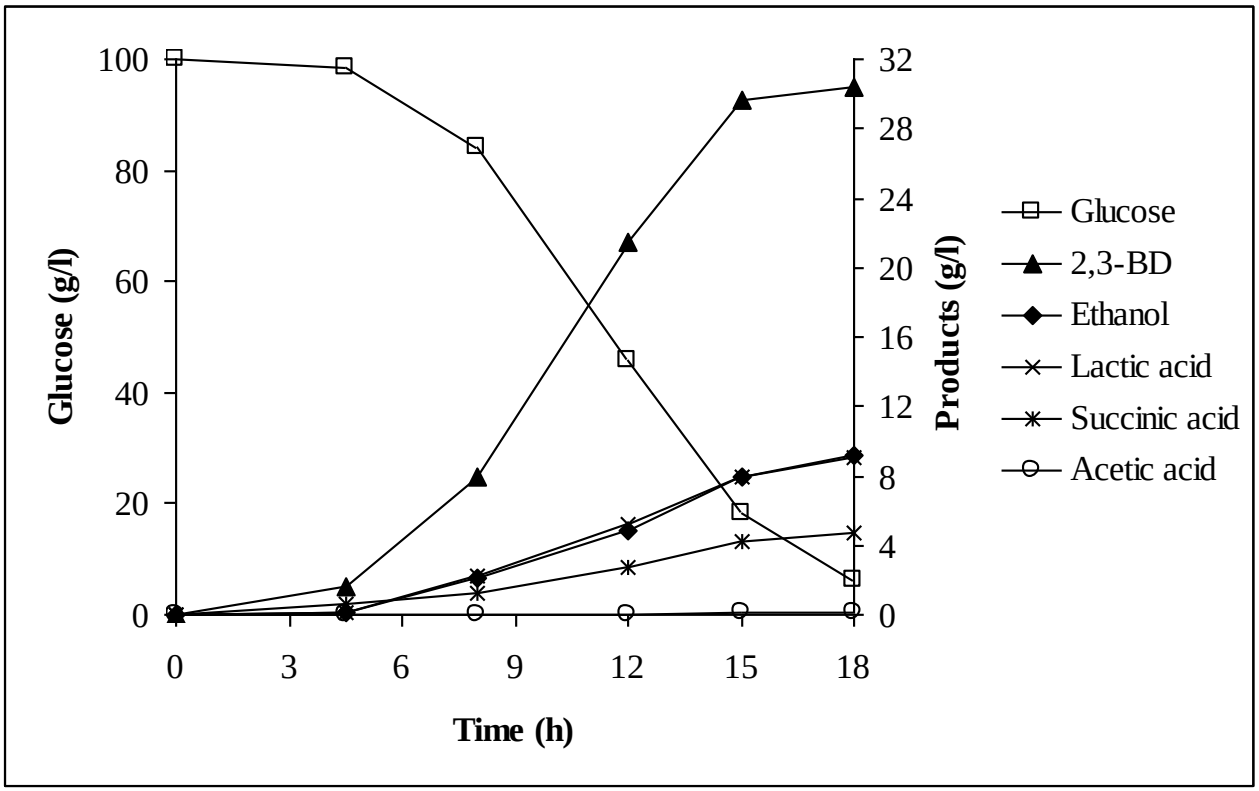


**Fig. 1.** Time courses of glucose consumption and products accumulation in batch fermentation. pH 6.0, air flow 1.45 vvm, agitation 500 rpm.

The titers of the soluble metabolites revealed that the highest aeration regime (air flow 1.6 vvm and agitation 700 rpm) is favorable only for biomass accumulation ($OD_{600}$=2.986 at 18 h). Glucose consumption completely ceased after few hours of fermentation, producing 2.08 g/l 2,3-BD and 1.5 g/l acetic acid. No other metabolites were detected. The highest concentration of 2,3-BD was obtained at an air flow of 1.45 (vvm) and agitation of 500 rpm – 24.15 g/l (productivity 1.34 g/l h) with an yield of 0.37 g/g glucose.

Batch fermentation in optimal conditions. Batch fermentation with presumed optimal pH and aeration was conducted (pH 6.0, air flow 1.45 vvm and agitation 500 rpm). After 18 h of fermentation 94 g/l glucose was consumed and 30.4 g/l 2,3-BD was accumulated (Fig. 1). The productivity was 1.69 g/l h and yield of 0.32 g/g glucose.

## DISCUSSION

The pH control and the aeration regime are the most significant factors determining the shift of the metabolic pathways in mixed acid fermentations. Most anaerobic processes are coupled with organic acids formation. Thus, in the course of the fermentation the medium acidifies and the growth and conversion of substrate gradually cease thus inactivating the culture by its own products [4].

Our observation is that extreme pH values are not suitable for 2,3-BD production. Considering the optimal pH for 2,3-BD production, the general opinion is that it is in the range between 5.0 and 6.0 [4,7,8]. Our results showed that at pH 7.0 the metabolic flux was shifted to acids production, especially in anaerobic conditions (Fig. 2).

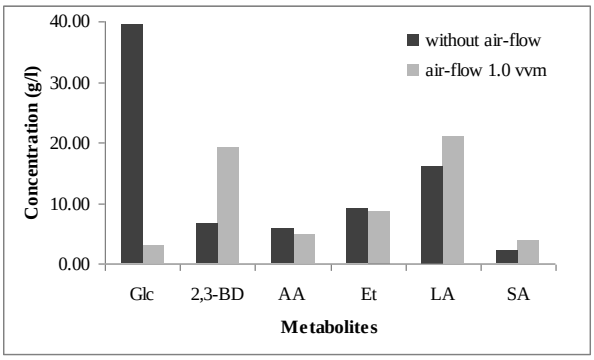


**Fig. 2** Comparison of the residual glucose and products formation in batch processes with or without aeration after 18 h of fermentation. Initial concentration of glucose – 100 g/l. Glc – glucose, 2,3-BD – 2,3-butanediol, AA – acetic acid, Et – ethanol, LA – lactic acid, SA – succinic acid.

In contrary, the decrease of the maintained pH of the medium led to diminishing of the acid production and enhanced the 2,3-BD yield. The conversion of glucose to 2,3-BD reached its maximum at pH below 5.5, but at the same time, the substrate consumption sharply decreased because of weak biomass formation. This is the reason for the final 2,3-BD titer decrease at pH above 6.0.

The moderate enhancement of the oxygen supply also turns the metabolic flux towards 2,3-BD. Results revealed that at the highest aeration regimes the consumption rate decreased and the carbon flux shifted only towards cells formation. The explanation is based on *Klebsiella pneumoniae* metabolism. This species is a facultative anaerobe and can get energy by two different pathways: respiration and fermentation [4]. When the oxygen supply is limited, both pathways are simultaneously active, therefore, its minimizing would increase 2,3-BD yield. On the other hand, when oxygen supply is too low, the accumulated biomass is negligible and this results in decreased yield of 2,3-BD. In anaerobic conditions, the strain cannot survive when pH is not controlled. The combination of low pH (below 4.8-5.0) and absence of oxygen is lethal for the culture (data not shown).

## CONCLUSIONS

Aiming at maximum 2,3-BD production, the optimum values of pH and aeration were investigated. The experimental results revealed that for *K. pneumoniae* G31, the maximum concentration and productivity (30.4 g/l, 1.69 g/lh) were obtained at pH 6.0, sterile air supply of 1.45 vvm and agitation speed of 500 rpm after 18 h of batch fermentation. These results are promising for 2,3-BD production on an industrial scale and prove the high potentialities of *K. pneumoniae* G31, comparable with the best known to date.

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# ВЛИЯНИЕ НА pH И АЕРАЦИЯТА ВЪРХУ ПОЛУЧАВАНЕТО НА 2,3-БУТАНДИОЛ ОТ ГЛЮКОЗА ЧРЕЗ *Klebsiella pneumoniae* G31

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(Резюме)

Щамът *Klebsiella pneumoniae* G31, екстра-продуцент на 2,3-butanediol (2,3-BD) от глицерол, е изпитан за ферментация при субстрат глюкоза. Оптимизирани са ключовите фактори, влияещи на ферментацията (pH и аерацията). Най-добрите условия, благоприятстващи получаването на 2,3-BD са намерени при pH 6.0, степен на аерация 1.45 vvm и обороти на бъркачката 500 об/мин. При това след 18 часа на ферментацияконцентрацията на 2,3-BD достига максимум от 30.4 g/l с производителност 1.69 г/л.чи добив от 0.32 г/г. Ефективността на процеса демонстрира потенциалното промишлено приложение на *K. pneumoniae* G31 като продуцент на 2,3-BD от глюкоза като суровина