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Effect of Different Carbon and Nitrogen Sources on the Growth of Newly Isolated *Clostridium* Strains and Butyric Acid Production

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Running title: **Butyric Acid Production by *Clostridium* via Different Carbon and Nitrogen Sources**

Abstract

In the recent years there is a growing interest in the fermentative production of butyric acid because of its wide applications in chemical, food, pharmaceutical, and fuel industries.

In this paper, the effect of different carbon sources, including glucose, arabinose, xylose, glycerol and glycerol + glucose, in view to increase the yield of butyric acid by 14 new isolated bacteria of the genus *Clostridium* was studied by batch fermentation. Similarly, peptone, yeast extract, meat extract, tripton, were added to the basic medium with optimal carbon source so that the best nitrogen sources were determined.

The results of this study showed that temperature of 37^0C, initial pH 7.5, glucose 20 (g/l) and a combination of nitrogen sources as follows: peptone (5 g/l), yeast extract (6.5 g/l), tryptone (2 g/l) led to higher butyric acid production.

Practical applications

The determination of optimum initial concentrations of carbon and nitrogen sources in the medium is an essential step in optimizing the fermentation process.

Butyric acid is widely used in food additives, pharmaceuticals, as well as a preservative, and therefore the natural origin is extremely important.

A subsequent optimization of the components of the medium and increasing the yield of the target product could be produced butyric acid by microorganisms that are not genetically modified.

Key words: butyric acid, *Clostridium*, fermentative production

Introduction

In the recent decades there is a strong interest towards production and use of organic products. Butyric acid (CH_3 CH_2 CH_2 COOH), is used in the chemical and food industries. It also plays an important role in the manufacture of plastic materials and in the textile industry. In the form of pure acid, it is used in the dairy industry to improve the butyric taste (Watson, 2002). Esters of the acid are used as additives to enhance the fruit flavors in the food industry and aromatics for the production of perfumes (Zigova et al, 2000; Vandak et al, 1997). Butyric acid finds particular application in the production of biodegradable polymers (Vandak et al, 1997).

Butyric acid derivatives are used for the production of anti-thyroid drugs and vasopressors used in anesthesia (Playne, 1985). Butyric acid is subject to increased research interest due to the its ability to inhibit tumor cells (Lupton, 2004), to neutralized activity of food carcinogens (Van Immerseel, 2010), and to lower cholesterol levels (Li, 2012). Currently butyrate is produced by petrochemical means, via the oxidation of butanaldehide derived from propylene by oxo process. Due to the low concentration of the final product biotechnological production is not competitive to the chemical production of butyric acid (Kroschwitz and Howe- Grant, 1993; Pryde, 1978). However, manufacturers of food and pharmaceutical products have requirements that nutritional supplements and medicinal products are produced by biological way, which requires the development of methods and strategies for optimizing the fermentation process (Hara, 2002, Vaseji et al, 2012).

The purpose of this study was to clarify the influence of the process conditions and of the various carbon and nitrogen sources on the production of butyric acid and the growth of new isolated microbial strains.

Materials and Methods

Microorganisms

In this work used 14 newly isolated strains identified as genus *Clostridium*, isolated from chickpea fermentation were used. Samples of chickpea from different geographical regions of Bulgaria were used for carrying out of the chickpea fermentation that was applied as starter in preparing special type of bread (simitt). The chickpea samples are selected from three geographical regions: Carnobat, Smyadovo and Dabene. Characterization of the isolated microorganisms from chickpea fermentation is done by methods as shown in Bergey's Manual [Whitman & Parte, 2009].

Long-term culture storage is carried out on MPB with 20% v / v glycerol at -20 ° C up to 4 months. Working cultures are stored in MPB medium at 4 ^{o}C and passaged every 20 days. Before starting the experiments, microbial cultures were passaged twice in nutrient broth. An inoculum was prepared by growing of 1 ml the culture in 10 ml sterile culture medium and incubated for 24 hours at 37 ^{o}C and anaerobic conditions achieved with Anaerocult® A (Merck Milipore, Germany). The thus prepared inoculum was used in all subsequent experiments.

Culture media

As a basic culture medium for the storage and maintenance of the strains and for determining the influence of the carbon source meat peptone broth (MPB) medium with composition (g / l): meat extract - 5; peptone - 15; NaCl - 5; K2HPO4 - 5 is selected.

To conduct the fermentation new isolated strains nutrient medium CM with composition (g / l): yeast extract - 5; K_2HPO_4 - 1; KH_2PO_4 - 1; $CaCl_2.2H_2O$ - 0,01; $(NH_4)_2SO_4$ - 0,1; $FeSO_4.7H_20$ - 0,005; $MgSO_4.7H_20$ - 0,1; pH = 7.5 was used. The medium was sterilized for 20 minutes at 121ºS.

Chickpea liquid medium (CLM) preparation: 1 part crushed chickpeas was mixed with 5 parts tap water and autoclaved at 1 atm for 30 min. The mixture was filtered through cheesecloth and the filtrate was centrifuged at 4000 rpm. / min. for 30 min. The pH of the separated supernatant was adjusted to 7.5 with 1N NaOH and was autoclaved again at 0.8 atm for 20 min.

The processes are carried out without pH control. As stated the initial pH is 7.5 for all processes.

Carbon source

The effect of different carbon sources was assessed by adding to the referred starting media (MPB) xylose, arabinose, glucose, glycerol at concentration of 20 g / l and a combination of glycerol and glucose (10 g / l each). For the fermentations with varying concentration of the carbon source the concentrations of the other

components in the media is constant. The initial pH was adjusted to 7.5 and the medium is distributed in 500 ml Erlenmeyer flasks (250 ml in flask) and sterilized. Culture media were inoculated and incubated at 37 ° C for 72 hours, the strictly anaerobic conditions are applied by means of Anaerocult A blocks (Merck). In the next step, the influence of the glucose concentration ranging in the amounts of 5, 10, 15, 20 and 50 g / l was studied.

Nitrogen source

The effect of different nitrogen sources in the production of butyric acid was investigated as to the production medium (C M) described above yeast extract, meat extract, peptone, tryptone were added. The sole carbon source in the medium is glucose at 20 g / l. Nitrogen sources were added to the medium in different combinations. The medium was sterilized, inoculated and incubated as described above.

Analytical methods

Butyric acid and glucose determination

The concentration of the target product and glucose was determined by HPLC. Samples were analyzed on a chromatographic system consisting of a pump Smartline S-100, Knauer, refractometric detector - Perkin - Elmer LC-25RI, column Aminex HPX- 87H, Biorad, 300x7, 8 mm and specialized software EuroChom, Knauer. 0,01 N H_2SO_4 was used as mobile phase, at a flow rate 0.6 ml / min.

Biomass concentration

For this purpose, a spectrophotometer SPEKOL 11, Carl Zeise, Jena was used. The concentration is determined by measuring the absorbance of the sample containing the biomass at a wavelength of 600 nM.

Results and Discussion

The influence of the type of carbon source on the development and production of butyric acid by 14 newly isolated strain of the genus *Clostridium* was studied. The results are presented in Figure 1A. It can be seen that the accumulation of the target product varies depending on the carbon source. In the fermentations carried out with substrate of glucose butyric acid concentration was at the highest level when strains 4A1 (2.31 g / l), 7a1
(2.38 g / l) and 8A3 (2.13 g / l), the pH of the culture medium drops to 4.5. Strains VT and 8A1 with glucose as carbon source do not produce the target product, but in combination of glycerol (10g / l) and glucose (10 g / l) the concentration of butyric acid was 1.92 g / l and 1.69 g / l respectively. The data presented in Figure 1.B. also show that strains 3RCM, VT, VTA, 3CD, 2622A assimilate xylose and arabinose as the produced butyric acid reached 2.3 g / l. Analysis of the results showed that, the ability to assimilate a certain carbon source vary depending on the strain. However, maximum productivity of butyric acid was observed in the presence of glucose, which confirms the general trend that this is the most commonly used carbon source in the medium for culturing microorganisms of the genus *Clostridium* [Al-Shorgani et al, 2011].

In the next step the effect of glucose concentration on the growth of strain 4A1, and on the production of butyric acid was studied.

Fig.2. shows the growth curve of strain 4A1, and the production of butyric acid during fermentation. As can be seen the strain has a well-defined exponential, starting from 25 hours and the stationary phase of 50 hours of the process.

From Fig.2 is seen that the accumulation of product should the growth curve. The production of butyric acid was associated with the growth of the strain by 64 hours of fermentation, reaching 3.2 g / l.

The substrate concentration varied as follows 5, 10, 15, 20 and 50 g / l. The results obtained are presented in Table 2. The highest yield of butyric acid (5,47g / l) was observed at 20 g / l glucose. The strain grew well and at substrate concentration of 15 g / l, the produced butyric acid was 4.95 g / l. When higher concentrations (50 g / l) was used, butyric acid production decreases, which may be due to catabolite repression. At the end of the fermentation process only 20% of the initial 50 g/l substrate is consumed.

The effect of different organic nitrogen sources combinations on the butyric acid production by means of strain 4A1 and the data are summarized in Fig.3. The best strain growth was observed under a combination of nitrogen sources as follows: peptone (5 g / l), yeast extract (6.5 g/l), tryptone (2 g / l). At this ratio and glucose concentrated of 20 g / l the concentration of butyric acid reaches 6.5 g/l.

Conclusions

In the present study it was found that the yield of butyric acid is at a high (5.95 g / l) with glucose (20

g / l) as carbon source and a combination of nitrogen sources as follows: peptone (5 g / l) yeast extract (6.5 g / l), tryptone (2 g / l).
Optimization of process parameters and adding in the midium of trace elements and growth factors is a prerequisite for increasing the yield of the target product and is subject to future investigations.

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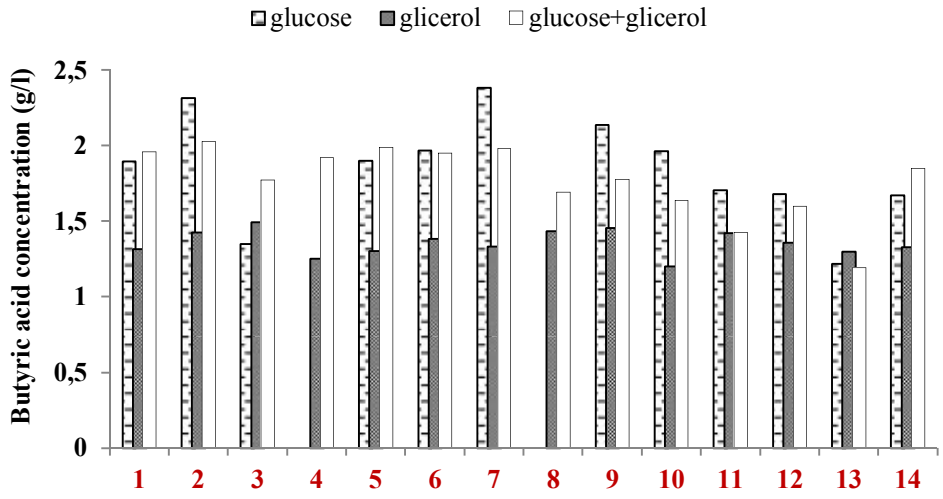

Fig.1A.

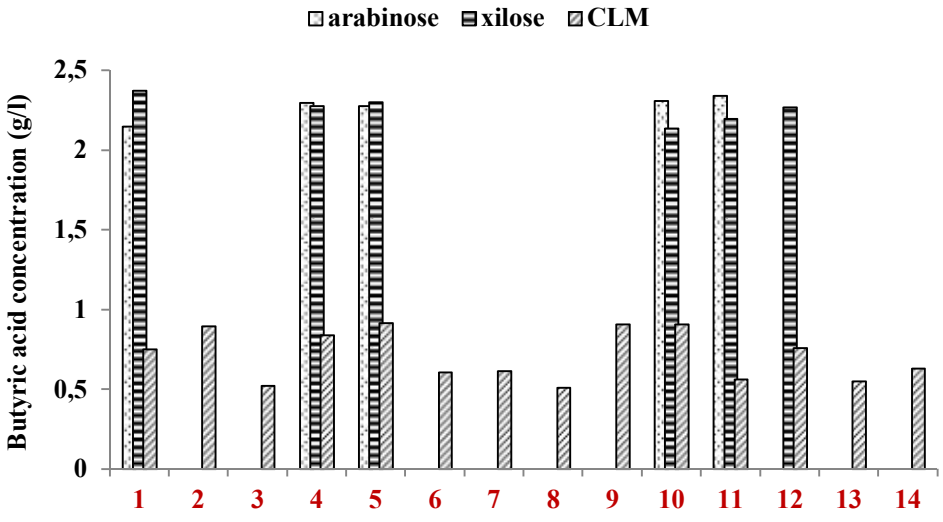

Fig.1B.

Figure 1 (A and B) Influence of different carbon sources on the butyric acid production at 37 ^{O}C and initial pH 7.5 at 48 h incubation
*1-**3RCM**; 2- **4A1**; 3-**4A3**; 4- **VT**; 5- **VTA**; 6- **6A1**; 7- **7A1**; 8- **8A1**; 9-**8A3**; 10- **3CD**; 11- **2622A**; 12- **5CD**; 13- **6CD**; 14- **2CD**;*

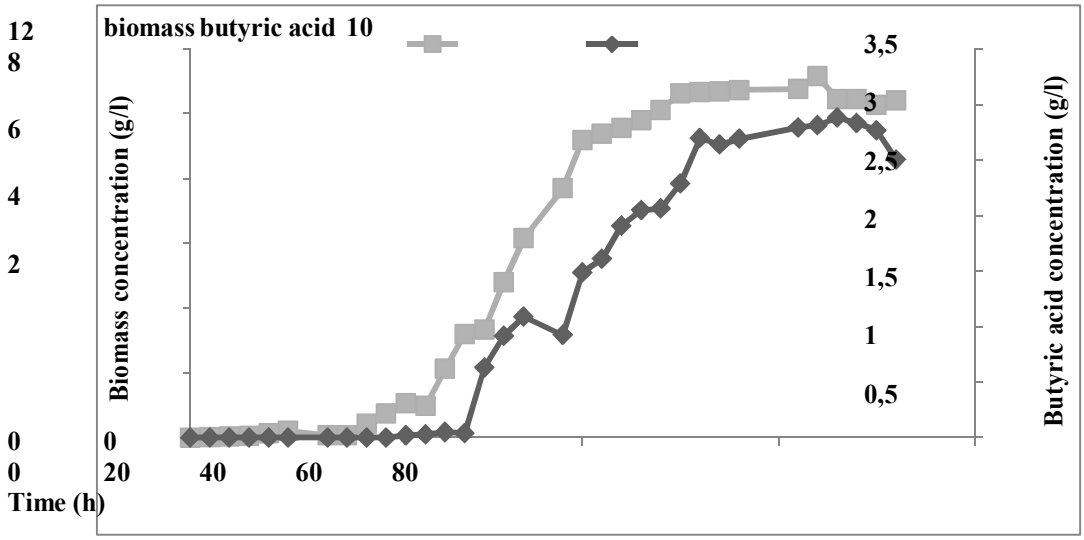

Figure.2. Growth of 4A1 strain and butyric acid production

Table 1. Influence of glucose concentration on the butyric acid production with 4A1 strain

Glucose (g/l)	Biomass (g/l)	Butyric acid (g/l)	C/N
5	4.97	1.25	0.734
10	7.43	3.67	1.468
15	10.41	4.95	2.202
20	13	5.47	2.936
50	6.52	2.45	7.339

Table 2. Combinations of different nitrogen source

	Pepton	Yeast extract	Tripton	Meat extract
CM1	10	3	-	10
CM2	2	5	2	-
CM3	2	6	2	-
CM4	5	6.5	2	-
CM5	5	2	2	-

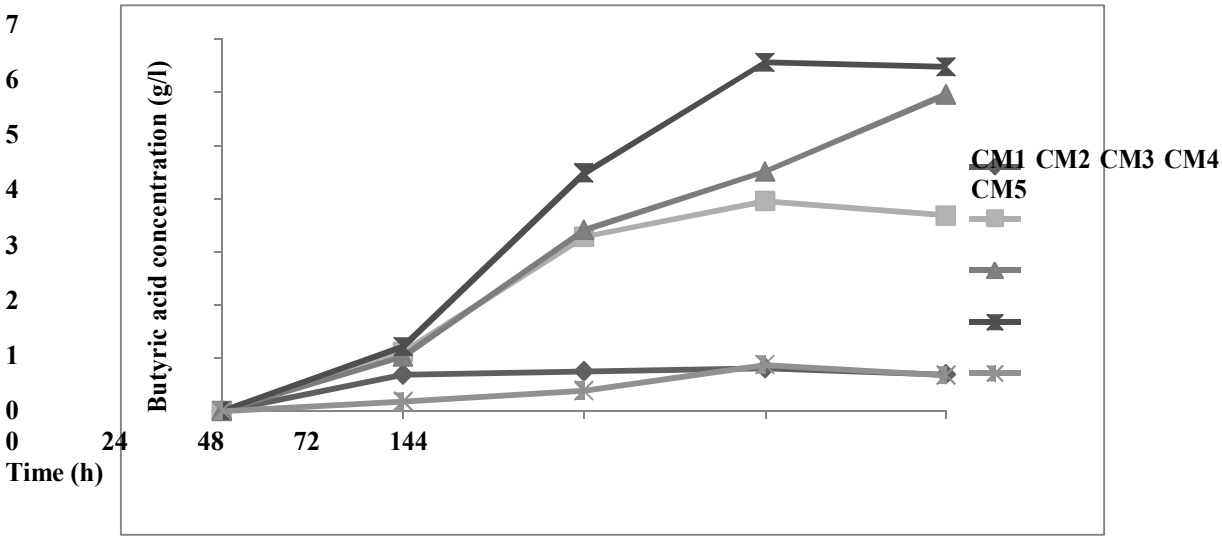

Figure 3.Influence of the type and concentration of the nitrogen source on the butyric acid production