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Isolation of *Clostridium acetobutylicum* ATCC824 Mutants Using Propionic and Isovaleric Acid Halogen Analogues as Suicide Substrates

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Abstract: The acetone-butanol-ethanol (ABE) fermentation process continues to attract attention as a potential feedstock for chemicals and liquid fuels. This fermentation is performed by solventogenic Clostridium species, such as *Clostridium acetobutylicum*, A possible way to improve the economic efficacy of acetone-butanol-ethanol fermentation is to increase the butanol ratio by eliminating the production of other by-products. This objective can be carried out by researches exploiting useful strains selected by genetic methods, especially mutagenesis. Thus, in the current study we use potassium salts of 3-bromopropionic, 2-bromopropionic, 2-bromoisovaleric and 2-chloropropionic acids as suicide substrates for *Clostridium acetobutylicum* mutant selection. Using nitrosoguanidine as mutagenic agent, 2-chloropropionate selected mutants altered in solventogenic phase while bromopropionate and 2-bromoisovalerate lead to obtain mutants with a butanol/acetone/ethanol ratio in favour of butanol production.

Key words: *Clostridium acetobutylicum* · Mutant · Suicide substrate · Fermentation

INTRODUCTION

Worldwide demand for energy has been the impetus for research to produce alcohol biofuels from renewable resources; particularly, the biosynthesis of butanol, which is regarded to be superior to ethanol as a fuel [1, 2]. Also, Production of alcohol from biomass is one way to reduce both consumption of crude oil and environmental pollution.

The metabolic pathway of *C. acetobutylicum* is of technical relevance because of the solvents it generates. The solvents acetone and butanol are bulk chemicals widely used in industrial applications, such as in paints and plastics. Acetone played a major role in the production of gun munitions during both world wars while butanol is currently being explored as a renewable alternative to petrol-based fuels.

In carbohydrate batch culture, *C. acetobutylicum* produces hydrogen, carbon dioxide, acetate and butyrate during a first growth phase. This results in a decrease in the pH of the culture medium. As the culture enters the stationary growth phase, the metabolism of the cells undergoes a shift to solvent formation and the bacterium produces butanol, acetone, ethanol, acetylmethylcarbinol and L-lactate [3]. Since, one of the main goals in biotechnology is the development of strains with enhanced yield of a desired product. fuel, several studies regarding solvent, particularly butanol production in *C. acetobutylicum* focused on different aspects to the ABE fermentation [1, 2, 4-7]. Thus, the ability to induce and isolate mutants has played a key role in the selection of industrially important strains. In the literature, several mutants of *C. acetobutylicum* have been described [8-15]. In other works, halogen derivatives was used as suicide substrates in *Clostridium acetobutylicum* and permitted to select mutant strains which were altered in their en-products compositions: 2-bromobutyrate leads to obtain strains deficient in acetone production [16], while bromoacetate, fluoroacetate, chloroacetate and 4-chlorobutyrate lead to obtain strains which lost solventogenic phase [17]. Hartmanis [18] has purified butyrate kinase from *C. acetobutylicum* and showed that the enzyme have affinity toward valerate, isobutyrate, propionate, isovalerate and vinylacetate and were 89%, 54%, 43%, 32%, 23% as effective as butyrate. This work describes end-products profile of mutants of

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C. acetobutylicum ATCC 824 isolated for resistance to bromo and chloropropionate halogen analogs and 2-bromoisovalerate.

MATERIALS AND METHODS

Micro-Organism: *C. acetobutylicum ATCC 824* was used in this study. The micro-organism was stored in Reinforced Clostridial Medium (R.C.M) at 4°C (Oxoid Ltd., Basingstoke, England).

Mutagenesis and Mutant Selection: Experimental studies were carried out in an anaerobic chamber (85% N_2, 10%CO_2 and 5% H_2) (Forma Scientific, Marietta, Ohio). Mutant selection was carried out in Petri plates containing the following compounds per liter of distilled water: K_2HPO_4.3 (H_2O), 0.5g; KH_2PO_4, 0.5g; $MgSO_4$.7 (H_2O), 0.2g; $FeSO_4$.7 (H_2O), 10mg; $MnSO_4$, 10mg; NaCl, 10mg; CH_3COO (NH_4), 2.2g; Glucose, 5g; Yeast extract (Difco Laboratories, Detroit, Michigan), 3g; Agar (Difco), 15g. This medium was adjusted to pH 5.2 with phosphoric acid and was supplemented with 1mg of 2-bromopropionate/l, 10mg of 3-bromopropionate/l, 10mg of 2-bromoisovalerate/l and 100mg of 2-chloropropionate/l (Potassium salts) (Aldrich Chemical Co., Milwakee, Wisconsin, U.S.A.). Petri plates were incubated with 100µl of an exponential culture growing on R.C.M. medium and a small quantity of N-methyl-N'-nitro-N-nitrosoguanidine (N.T.G.) (Aldrich Chemical) was placed on the centre of each plate. After 72 hours of incubation at 35°C, colonies resistant to these halogen derivatives were inoculated on R.C.M. medium for further study.

Fermentation Medium: The pre-culture medium contained the following components per liter of distilled water: $(NH_4)_2SO_4$, 3g; K_2HPO_4.3 (H_2O), 0.5g; $MgSO_4$.7 (H_2O), 0.2g; $FeSO_4$.7(H_2O), 10mg; $MnCl_2$.4(H_2O), 10mg; $(NH_4)6Mo_{24}$.4 (H_2O), 10mg; Glucose, 60g; Yeast extract, 4g; $CaCO_3$, 3g. This medium without CaCO3 was used as the culture medium. An exponential culture grown on the pre-culture medium was used as inoculums (10% V/V) in a 2 liter fermentor (Biolafitte). The growth temperature was 35°C.

Analysis Methods: Cells concentrations were estimated by cells dry weight measurement using a predetermined correlation between absorbance at 600 nm and cells dry weight. Analysis was made on supernatant of the sample previously centrifuged at 10000g for 15 min. at 4°C. Concentration of residual glucose was determined by the method of Miller *et al*. [19]. Fermentation products (ethanol, acetone, acetate, butanol and butyrate) were determined by gas chromatography as described previously [20]. Acetylmethylcarbinol (acetoin) was determined according to the procedure of Westerfeld [21] and L-lactate by Boehringers Kit (Boehringer Mannheim, Mannheim, Germany).

RESULTS

The major characteristics of mutants described herein are summarized in Table 1. Mutants isolated for resistance to 2-chloropropionate exhibit a classic acidogenic course profile: these mutants convert only 23g of glucose/l (instead of 64g /l in the parent strain) into acids as the major end-products (the percentage of acid conversion is 37.4% and that of solvent is 4.7% in mutants instead of 3.1% and 28% respectively in the wild type strain). Mutants isolated for resistance to bromopropionate and bromoisovalerate exhibit similar fermentation course profile. These mutants convert 47g of glucose /l and are altered in the ethanol and acetone production (0.6g of ethanol/l and 0.8g d'acetone/l are produced at the end of the fermentation in mutants instead of 2g/l and 3g/l respectively in the wild type). The percentage of the ethanol/glucose and that of acetone/glucose conversion are 1.27% and 1.7% in the mutants versus 3.1% and 4.7% in the parent strain), whereas the percentage of butanol/glucose conversion at the end of the process is still nearly the same (21.27% in the mutants and 20% in the parent strain). However, mutants accumulate acetate (4g/l versus 1.5g/l in the parent strain), produce more lactate (0.5g/l versus 0.04g/l in the parent strain) and more acethylmethylcarbinol (acetoin) than the parent strain (3.6g/l versus 1.5g/l) (Table 2). Biomass formation and butyrate uptake are almost identical (Fig. 1 and Fig. 2).

Table 1: Fermentation end products after conversion of glucose by *Clostridium acetobutylicum* parent strain and mutant strains isolated for resistance to some butyrate kinase halogen analogue substrates

	End products (g/l)				
Mutants resistant	Ethanol	Acetone	Butanol	Acetate	Butyrate
3-bromopropionate					
2-bromopropionate	0.6	0.8	10	4	1.3
2-bromoisovalerate					
Yields (%)	1.7	1.7	21.27	8.5	2.7
2-chloropropionate	0.1	0.11	0.88	3.4	5.2
Yields (%)	0.4	0.48	3.82	14.8	22.6
Parent strain	2	3	12.8	1.5	0.5
Yields (%)	3.1	4.7	20	2.3	0.8

Table 2: Acetoin and L-lactate production (g/l) by *Clostridium acetobutylicum* parent strain and mutant strains isolated for resistance to bromo-derivatives of propionate and isovalerate

	Acetoine	L-lactate
Parent strain	1.5	0.04
Mutant strains	3.6	0.50

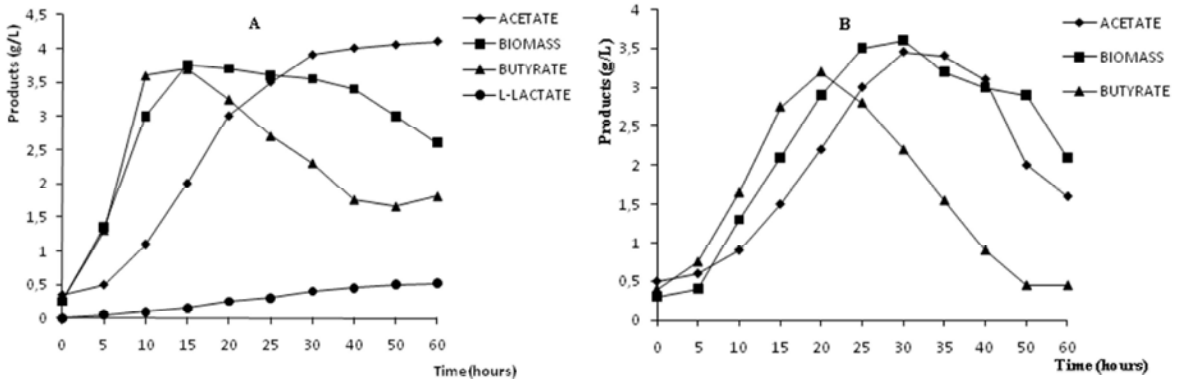

Fig. 1: Course of acids and biomass production by *Clostridium acetobutylicum* ATCC824 (A) Mutants strains and (B) wild type strain growing on glucose containing media

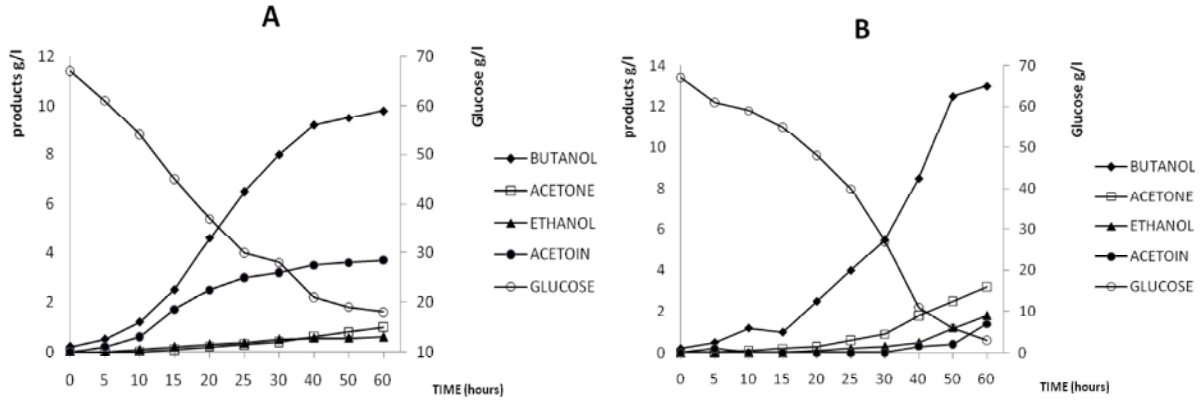

Fig. 2: Course of solvents production by *Clostridium acetobutylicum* (A) mutant strains and (B) wild type strain growing on glucose containing media

DISCUSSION

The N.T.G. mutagenesis technique and the use of 2-bromopropionate, 3-bromopropionate and 2-bromoisovalerate as suicide substrates provided mutants which are altered in the yields of ethanol and acetone production while that of butanol was unaffected. Similar results have been obtained, by using 2-bromobutyrate as suicide substrate where the acetone pathway was completely abolished leading to a butanol/acetone/ethanol ratio of 2.5/0/1 [16]. Acetate uptake was no longer occurred during the solvent phase leading to acetate accumulation, whereas the butyrate reutilization lead to butanol production without intermediate as suggested by Hartmanis [18]. Thus, our results confirm the fact that acetone and butanol pathways in *C. acetobutylicum* are not coupled and a possible butyrate-butanol bioconversion pathway still to be investigated. During fermentation by *C. acetobutylicum* strains, butanol is the most attractive end-product and the mutants described in this paper showed that it is possible to shift the butanol/acetone/ethanol ratio wildly in favour of butanol which becomes 16.7/1.3/1 instead of 6.4/1.5/1 in the wild type. In fact, butanol is considered a promising product of biomass fermentations for potential industrial use as a solvent, chemical feedstock and particularly liquid fuel. Therefore, it is very important to optimize the product yield and simultaneously reduce the level of other end products.

However, to maintain the oxido-reduction balance, mutants isolate in the present study produce more acethylmethylcarbinol and more L-lactate than the parent strain.

Our results and those reported elsewhere [16, 17] showed that selection for resistance to halogen analogue substrates provided a successful tool to isolate mutant strains of *C. acetobutylicum* for physiological studies, genetic studies and industrial process.

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