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Enhanced Butanol Production by Mutant Strains of Clostridium acetobutylicum in Molasses Medium

[Melaslı Ortamda Mutant Clostridium acetobutylicum Suşlarının Butanol Üretiminde Artış]

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ABSTRACT

The present study deals with the enhancement of n-butanol production by a mutant strain of *Clostridium acetobutylicum* using blackstrap molasses as substrate (a by product of sugar industries). The bacteria were cultured in 14 L glass stainless steel fermentor. The parent strain *Cl. acetobutylicum* PTCC-23 was mutagenized for the improved production of butanol by UV exposure, N-methyl-N-nitro-N-nitrosoguanidine and ethyl methane sulphonate treatments. After extensive screening and optimization, a total of 15 different mutants were selected. The best butanol producing strain was designated as MEMS-7, which yielded 20 % more butanol compared to parent strain after 91 h of incubation at 30°C. The optimum sugar level in molasses medium was 60.0 g/L. The growth of the parent strain PTCC-23 was inhibited at 1.6 mol/L butanol. The butanol tolerance of the mutant strain MEMS-7 on the other hand was found to be 3.84 mol/L. The fermentation profile and kinetics study indicate the superiority of the mutant strain for n-butanol production from blackstrap molasses.

Key Words: *Clostridium acetobutylicum*, blackstrap molasses, anaerobic fermentation, butanol, mutation, kinetics assessment

ÖZET

Bu çalışma şeker endüstrisinin yan ürünü olan melası substrat olarak kullanan mutant *Clostridium acetobutylicum* suşunun n-butanol üretimindeki artışla ilgilidir. Bakteri 14 L cam paslanmaz çelik fermentörde üretildi. *Cl. acetobutylicum* PTCC-23 butanol üretimini arttırmak için UV ışığı, N-metil-N-nitro-N-nitrosoguanidin ve etil metan sulfonat ile muamele edildi. Yoğun tarama ve optimizasyon çalışmaları sonucu 15 farklı mutant seçildi.

En iyi butanol üreten MEMS-7 suşu, 30 °C'da 91 saat inkübasyondan sonra % 20 fazla butanol üretti. Melaslı ortamın optimum şeker düzeyi 60.0 g/L idi. Ana suş PTCC-23'ün büyümesi 1.6 mol /L butanol tarafından inhibe edildi. Diğer yandan mutant suş MEMS-7'nin butanol toleransı 3.84 mol/L olarak bulundu. Fermentasyon profile ve kinetic çalışmalar mutant suşun melastan n-butanol üretimindeki üstünlüklerini gösterdi.

Anahtar Kelimeler: *Clostridium acetobutylicum*, blackstrap melas, anaerobik fermentasyon, butanol, mutasyon, kinetik çalışma.

INTRODUCTION

The microbial fermentation of carbohydrates to butanol is well known (1). Currently, this value-added fermentation is viewed as potentially attractive for several economic reasons. Predominant among economic factors is the current surplus of agricultural waste or by-products that can be utilized as inexpensive fermentation substrate. In Pakistan 700000 tons of molasses are produced annually. Therefore, it is an ideal place for the development of fermentation technologies. The fermentation method for butanol production does not compete economically with the petrochemical route because it yields a very dilute product and posess a high energy demand for its recovery (2). The primary reason for low butanol concentration is its toxicity. Butanol when present at 13 g/L in the fermentation medium becomes toxic to the culture due to its hydrophobic nature (3). Therefore, it is essential to find bacterial strains with higher butanol tolerance. Isolation of mutants has played a traditional key role in the selection and improvement of industrially important strains (4). The solvent yield is reported to increase by mutant strains, which can tolerate high butanol concentrations. Attempts to increase butanol productivity were made by various workers (5,6,7). Likewise, the present study was undertaken to develop the mutant strains of *Cl. acetobutylicum* with enhanced butanol tolerance and productivity in anaerobic fermentation by blackstrap molasses as substrate.

MATERIALS AND METHODS

Organism

The strain of *Cl. acetobutylicum* PTCC-23 was obtained from Pakistan Type Culture Collection (PTCC). The spores were stored at 4 °C in RCM (Reinforced Clostridia Medium, Oxoid) under strict anaerobic conditions (Anaerobic jar-Oxoid). TGY medium containing (g/L): 10.0 tryptone, 6.0 glucose, 6.0 yeast extract, 2.5 KH_2PO_4, 0.25 $MgSO_4.7H_2O$, 0.035 $Na_2S_2O_4$ and 0.003 resazurine was used as a basal medium (8).

Mutagenesis

The mutant strains of *Cl. aectobutylicum* were derived from parent strain PTCC-23. The stock cultures were maintained on TGY medium at 1.0 x 10^6 cells/mL for U.V. irradiation and further studies. The medium, buffer and distilled water were placed in anaerobic chamber at least 24 h before use to ensure the removal of dissolved oxygen. The dose of UV exposure given to bacterial suspension was 1.2 x 10^6 J/m^2/S for different time intervals (5 to 60 min). Survival curve was prepared and time of exposure giving (1.0 x 10^3 CFU/mL) 3 log-kills was used for the selection of mutants. Chemical mutagenesis was carried out with N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and ethyl methane sulphonate (EMS) by following the modified method of Rogers and Pallosari (9). The treatment was given with chemical mutagens for a period of 55 to 60 min, at concentration of 1.0 mg/ml in cold tris-acetate buffer (pH 6.5) containing 0.1 % sodium thioglycolate. The survival was less than 2 %. All the putative mutants were selected and characterized in fermentation medium containing 120 g/L of molasses. The best mutants were selected for maximum production of butanol.

Growth inhibition studies

Growth inhibition of parent and mutant strains by butanol was studied by culturing the bacteria in TGY medium. Different concentrations of butanol ranging from 0.48 to 4.32 mol/L were added to solid culture media preparations. The bacteria were enumerated after incubation at 32 ± 2°C for 48 h. All trials were conducted in triplicate.

Molasses clarification

Blackstrap molasses obtained from Pattoki Sugar Mills, Pakistan, was clarified according to the method of Panda *et. al.* (10). Forty mL of 0.1 N H_2SO_4 was added to 1 L molasses medium with 50 % sugar content. The medium was kept at 80°C for 1 h in water bath and then neutralized with $CaCO_3$. After 24 h, sugar contents of clear supernatants were adjusted to 12 %.

Fermentation technique

The fermentation medium was composed of (g/L): 120 (6 % sugar) cane molasses, 3.0 $(NH_4)_2SO_4$, 0.7 super phosphates, 2.0 $CaCO_3$, at pH 6.2 (8). The vegetative inoculum was developed in test tubes containing 15 mL of medium. The tubes were incubated in anaerobic jars under hydrogen gas at 32 ± 2°C for 24 h. The size of the inoculum was further increased by inoculating the fermentation medium at rate of 3 % (v/v) in 250 mL and 1 L flasks, containing 200 mL and 800 mL of fermentation medium, respectively. The inoculum was transferred aseptically by gravity feeding into 9.0 L (working volume) sterilized fermentation medium in the 14.0 L (capacity) glass stainless steel fermentor (Marubishi-Japan). The sterilized slurry of $CaCO_3$ was added aseptically into the fermentation medium. The initial medium pH at 6.2 was not regulated throughout the fermentation. Hydrogen gas was spurged for anaerobic condition. The fermentation was carried out at 32 ± 2°C for 96 h. The butanol was recovered from the broth by distillation.

Analytical methods

Cell concentration was estimated by cell dry weight measurement using a predetermined correlation between optical density at 600 nm and cell dry weight (11). Analyses were made on supernatant of samples centrifuged at 10000 x g (Eppendorf 5804 R, Fixed-angle rotor F-34-6-38) for 10 minutes. Total sugar in fermentation medium was analyzed by dinitro salicylic acid method (12). Butyrate and butanol were determined by GLC. The analysis of the chromatographic data was carried out by using ICR IB integrator (Intersmet, France). All the experiments were conducted in triplicate and the results were expressed as mean value with their standard errors.

Kinetics analysis

Rate of cellular, sugar uptake, acid (butyrate) and solvent (butanol) formation were expressed as g/L/h. The cell mass formation and sugar uptake rates were determined as a slop of the tangent to the experimental curve during the course of fermentation. Similarly acid (butyrate) and solvent (butanol) formation rates were measured. The specific rates of substrate uptake and product formation were given as described by Fond and Engasser (11).

RESULTS AND DISCUSSION

In designing a mutation protocol for a particular mutagen, it is important that an effective mutagen be chosen. As presented in Table 1, UV irradiations were found to be ineffective in increasing the butanol production. It is supposed to be due to that *Cl*. species may be deficient in error prone repair required for indirect mutagenesis (4). The N-methyl-N-nitro-N-nitrosoguanidine (MNNG) mutants, on the other hand, exhibited insignificant increase in butanol productivity compared to parent strain under the test conditions used. However, the best mutant strain named MEMS-7 was obtained after the treatment with ethylmethane sulphonate (EMS). It gave 20 % higher butanol production. Lammel (13) also failed to induce mutation with N-methyl-N-nitro-N-nitrosoguanidine (MNNG).

The important key factors which determine the economic viability of butanol fermentation is the cost of raw material which make up about 60 % of the overall cost (14). Therefore, the initial level of sugar in molasses medium was optimized. Table 2 shows that the mutant strain MEMS-7 gave maximum yield of butanol in the medium containing 120 g/L of molasses (6 % sugar). A gradual reduction in the solvent yield was observed with the increase in the sugar concentration of the molasses medium due to metabolic inhibition (11). It has been described that acidogenic phase is dominant at low sugar concentrations (15).

The mutant strain MEMS-7 showed enhanced butanol productivity in terms of yield coefficients, Yp/s, Yp/x and Yx/s, as mentioned in Table 3 compared to parent strain and other selected mutants. Product yield coefficients (Yp/s and Yp/x) 0.37 and 6.7 (g/g) were improved as compared with the rest of strains. All the parameters for butanol production are much improved by best mutant strain MEMS-7 strain as described in Table 4. Significant specific butanol productivity (0.09) was observed with MEMS-7 mutant.

Figure 1 shows that both parent and mutant strain, MEMS-7 exhibited the acidogenic phase followed by solventogenic phases during the entire course of fermentation. The earlier accumulation of high amount

Table 1. Comparison of butanol productivity by mutant strains with parental strains of Cl.acetobutylicum in laboratory scale fermenter

***Cl. acetobutylicum* strains**	**Maximal bio-mass** (g/L)	**Total sugar fermented** (g/L)	**Butanol Conc.** (g/L)	**Cellular morphology**
Parent strain				
PTCC-23	3.10±0.246	51.01±0.284	15.01±0.257	compact, smooth, regular
UV irradiated mutants				
MUV-1	2.90±0.330	49.31±0.264	15.12±0.348	compact, smooth, regular
MUV-2	2.52±0.210	47.35±0.318	14.82±0.263	compact, smooth, regular
MUV-3	2.45±0.271	48.71±0.253	15.03±0.337	compact, irregular, raised
MNNG treated mutants				
MNNG-1	2.84±0.304	51.21±0.343	15.34±0.347	compact, smooth, regular
MNNG-2	3.52±0.316	54.37±0.317	15.72±0.252	compact, raised , regular
MNNG-3	2.30±0.158	48.71±0.192	14.40±0.296	compact, smooth, irregular
MNNG-4	2.70±0.275	52.04±0.261	15.45±0.333	compact, raised, regular
EMS-treated mutants				
MEMS-1	2.74±0.305	51.71±0.308	15.30±0.251	compact, smooth, regular
MEMS-2	2.43±0.281	48.32±0.268	15.72±0.183	smooth, regular, translucent
MEMS-3	2.61±0.192	53.0±0.287	14.30±0.189	compact, smooth, regular
MEMS-4	2.34±0.264	47.8±0.171	15.60±0.255	smooth, regular, viscous
MEMS-5	2.81±0.272	51.70±0.349	16.01±0.325	raised, regular, viscous
MEMS-6	2.92±0.247	53.21±0.274	17.20±0.281	compact, smooth, creamy
MEMS-7	2.70±0.309	49.01±0.285	18.02±0.257	compact, smooth, irregular
MEMS-8	3.13±0.197	54.50±0.198	16.47±0.328	compact, smooth, irregular

All the experiments were carried out at 30°C following growth on 60 g/L initial sugar level. The initial pH of the molasses medium was 6.2, which was not regulated throughout the fermentation period of 96 h. Values followed by ± are standard errors of mean.

Table 2. Effect of different concentration of molasses sugar on butanol productivity by mutant stain of *Cl. acetobutylicum* MEMS-7

Molasses Sugar (g/L)	Maximal biomass (g/L)	Maximal butyrate conc. (g/L)	Sugar consumed (g/L)	Butanol production (g/L)
20.0	1.23±0.257	2.40±0.348	32.21±0.342	7.13±0.187
40.0	1.83±0.264	3.25±0.270	43.12±0.317	13.21±0.262
60.0	2.81±0.319	4.20±0.322	49.30±0.285	18.03±0.257
80.0	3.05±0.331	4.73±0.338	52.41±0.312	12.04±0.329
100	3.37±0.328	5.23±0.343	54.45±0.329	11.35±0.337

Initial pH 6.2, incubation temperature 30°C for 96 h in 14 L glass stainless steel fermentor (working volume, 9 L). Values followed by ± are standard errors of mean.

of butyrate by MEMS-7 over parent strain may be a major factor in the improved production of butanol by the mutant strain, due that higher concentrations of acid (butyrate) increase the solvent yields (16,17). Moreover, the mutant strain MEMS-7 not only produced 20 % more butanol but the solvent formation set sooner than that of the parent strain. The faster onset of solvent formation by strain MEMS-7 has important economic implication as the fermentation time reduced by 5 h. The table 5 depicts a distinct inhibitory effect of n-butanol on the growth of strains PTCC-3 and MEMS-7. The growth of the parent strain was completely inhibited at 2.4 mol/l butanol whereas the mutant strain showed positive growth even at 3.82 mol/L butanol. The results of the present study are in good agreement with earlier investigators (18) reporting the isolation of a tolerant strain of *Cl. aectobutylicum* producing 13 % more butanol than the parent strain in extruded corn broth. The butanol tolerance of the mutant strain MEMS-7 was higher than the strains reported earlier (19,20,21). According to Shirley *et. al.* (2) the enhanced butanol productivity by mutant strain was due to the adaptation process involving modification of the lipid composition of the bacterial cell membrane which became less sensitive to fluidizing effect of butanol. The production of valuable industrial solvent (butanol) by anaerobic fermentation of blackstrap molasses appears to be highly economical due to the availability of the least expensive substrate and relatively high yield of the product.

The authors concluded that the best-selected mutant strain exhibited high butanol tolerance in the optimized molasses medium leading towards enhanced productivity

Table 3. Comparison of butanol productivity among parent and best mutants of *Cl. acetobutylicum*

Strain tested	Butanol production (g/L)	Kinetic parameters		
		Yp/s	Yp/x	Yx/p
PTCC-23	15.02±0.280	0.345	5.00	0.60
MEMS-6	17.02±0.281	0.324	5.93	0.054
MEMS-7	18.10±0.257	0.367	6.66	0.055
MEMS-8	16.47±0.328	0.306	5.61	0.55

Yp/s (g/g) = butanol produced (g/L)/substrate consumed (g/L), Yp/x (g/g) = butanol produced (g/L)/cell mass formed (g/L), Yx/s (g/g) = Cell mass formed (g/L)/substrate consumed (g/L). Values followed by ± are standard errors of mean.

Table 4. Comparison of kinetic parameters for production of butanol from 120 g/L molasses carbohydrate with parent strain of Cl. acetobutylicum PTCC-23 and its best mutant derivative

Kinetic Parameters	Parental strain PTCC-23	Mutant strain MEMS-7
Substrate consumption parameters		
μ (h^{-1})	0.127	0.123
Qs (g/L/h)	0.61	0.56
qs (g/g cells /h)	0.197	0.214
Product formation parameters		
Qp (g/L/h)	0.182	0.24
Qp (g/g cells/h)	0.058	0.090

Kinetic parameters: μ (h^{-1}) = specific growth rate, Qs = substrate consumed/L/ h, qs = g substrate consumed /g cells /h, Qp = g butanol produced /g cells/ h

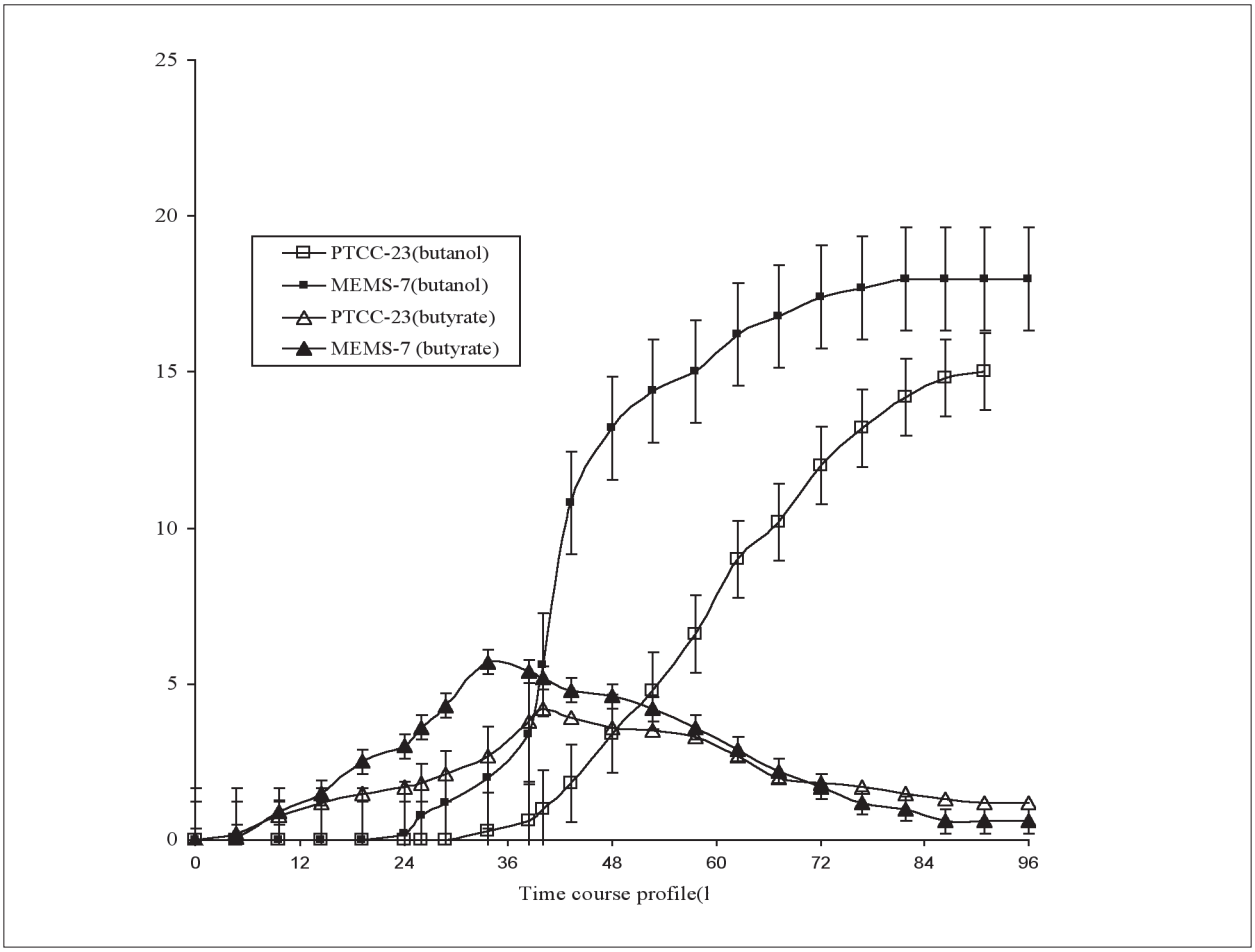

Figure 1. Time course comparision of parental PTCC-23 with the best mutant MEMS-7 for butyrate butanol productivity. All fermentations were carried out at 32°C for 96 h, following growth on 60 g/L sugar concentration and initial pH at 6.2.

Table 5. Growth response of Cl. acetobutylicum strain PTCC-3 and MEMS-7 to butanol

Butanol conc. (mol/L)	**PTCC-23** (cells/mL)	**MEMS-7** (cells/mL)
0	50 x 10^6±1527525.232	70 x 10^6±1154700.538
0.48	10 x 10^5±25166.115	16 x 10^6±371184.2909
0.96	80 x 10^3±2081.666	10 x 10^5±15275.253
1.44	720±25.166	2.0 x 10^4±513.160
1.92	49±0.577	156 x 10^2±737.111
2.40	0	46 x10^2±251.66
2 .88	0	310±15.275
3.36	0	72±2.5167
3.84	0	39±0. 2.309
4.32	0	0

Cell number was counted after 48 h of incubation on YGM medium containing different levels of butanol. Values followed by ± are standard errors of mean.

of butanol. By quantitative analysis of product formation the strain MEMS-7 can be exploited on pilot scale to cope with the increasing demand of butanol in Pakistan as well as in other beet sugar producing countries.

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