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Isolation and characteristics of hyper-butanol producing OBT7 mutant of *Clostridium saccharoperbutylacetonicum* N1-4

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Abstract : 1) OBT7 mutant was isolated by UV light-butanol tolerance from *Clostridium saccharoperbutylacetonicm* ATCC 13564 (N1-4 strain). The mutant produced 16.5 g/*l* (1.4-fold increase) of n-butanol, 4.65 g/*l* (1.5-fold increase) of acetone, and 21.5 g/*l* of total solvent. It was suggested that clostridial bacteria producing n-butanol does not have a poor effect on misrepair *via* an error-prone pathway by UV light-butanol tolerance. 2) Compared to glucose fermentation, in mannitol fermentation, OBT7 mutant did not produce acetone and acetic acid. And the ratios of n-butanol and ethanol to total solvents increased by 10.3% and 10.5%, respectively, totalling 20.8%, while the ratio of acetone was decreased by 21.2%. Also the maximum ratio of n-butanol to total solvents reached 94.8%. These results indicated that oxidized compound (acetone, acetic acid, and butyric acid) was converted to the reduced compounds (n-butanol, and ethanol). Therefore, mannitol can be used to eliminate by-products of oxidized compound(Received November 2, 1992; accepted January 15, 1993).

For improved solvent production, isolation of chromosomal auxotrophic mutants from clostridia is difficult.[1] Only a few auxotrophic mutants of *Clostridium* have been obtained for genetic studies: mutant strains of *Clostridium thermocellum* and *Clostridium pasteurianum* have been isolated by various mutagens such as UV light,[2,3] *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine(MNNG),[2,4] and ethyl methan sulfonate (EMS)[2,5] as well as by spontaneous mutation [2,6], and mutant of *Clostridium acetobutylicum* and *Clostridium saccharolyticum* have been isolated by only EMS,[7,8] spontaneous mutation[9] and MNNG,[10] respectively. Compared to thermophilic clostridia, the mutagenesis of *C. acetobutylicum* has been made difficult by MNNG or UV light which is well known as a poor mutagen for misrepair error-prone process.[11]

This paper reports the isolation of clostridial mutant producing hyper-solvents (butanol, ethanol, acetone) by UV light-butanol tolerance and the elimination of by-product such as acetic acid, butyric acid, and acetone using mannitol as a sole carbon source with the hyper-butanol producing mutant derived from *Clostridium saccharoperbutylacetonicum* ATCC 13564 (N1-4 strain).

Materials and Methods

Microorganisms

The microorganisms used were hyper-butanol producing OBT7 mutant derived from *Clostridium saccharoperbutylacetonicum* ATCC 13564 (strain N1-4) and the parent N1-4 strain. The spores of OBT7 mutant and N1-4 strain were used as inocula as described by Hayashida and Ahn.[12]

Media

The composition and the preparations of PG medium and TYA medium were described by Hayashida and Ahn.[12]

Key words : *Clostridium saccharoperbutylacetonicum*, mannitol fermentation, acetone, butanol

Induction and isolations of mutant

Induction and isolation were done as described by Hayashida and Ahn.[12] For the isolation of UV light-butanol tolerant mutant, after N1-4 strain was irradiated UV light, the cell was proceeded by method of a serial stepwise procedure of n-butanol (0~20 g/*l*).

Fermentation process

1) Pre- and main culture were followed as mentioned by Hayashida and Ahn.[12] The main culture was done in 100, 400 and 500 m*l* TYA media containing desired quantitative glucose or mannitol as the carbon source as static culture at 30℃. 2) Inocula were used with 10% (v/v) unwashed cells from preculture. But mannitol fermentation was done by 10% of cells which were washed twice with 0.15 M NaCl by centrifuging at 3019×g for 5 min.[13]

Methods of analysis

Sampling interval was 2 h on each fermentation by OBT7 mutant or N1-4 strain. Fermented cultures were centrifuged (Model KR-20000, Kubota Co., Japan) at 9800×g for 15 min at 4℃. The supernatants were used for analyses. 1) Cell mass concentration[14] was measured by dry weight of the bacteria. The procedure of harvested cell crops was as follows; The pellet sediment was washed twice with 0.15 M and 0.015 M NaCl by centrifuging (3000×g, 5 min), respectively, as above. The final sediment was suspended with 1 N acetic acid to neutralize $CaCO_3$ and filtered through cellulose nitrate membrane filter (pore size 0.45 micrometer, dia. 7 mm; type TM-2; Toyo Roshi Co. Ltd., Japan).[13] For the cell mass concentration, the harvested cells were dried at 60℃ for 24 h. 2) Reducing sugar was measured by the method of Somogyi-Nelson.[15] 3) Solvents (ethanol, acetone, and n-butanol) and acids (acetic acid, and n-butyric acid) were analyzed by gas chromatography (Model 163; Hitachi Ltd., Japan) equipped with a flame ionization detector as described by Hayashida and Ahn.[12] 4) Collection and composition of the gases (H_2 and CO_2) were done as described by Ahn and Hayashida.[16]

Results and Discussion

Selection of mutant

OBT7 mutant was isolated from *C. saccharoperbutylacetonicum* ATCC 13564 (strain N1-4) by UV light-butanol tolerance And the mutant could not be obtained from only UV light or butanol tolerance or the other mutagen. The mutant was selected from 4,000 colonies from UV irradiation at 253.6 nm, distance 30 cm, 20 sec. Survival rate of the mutant 34×10^{-2}%. And the mutant tolerated with 0~20 g/*l* of butanol. The mutant formed a spore.

Anaerobic bacteria, especially, *C. acetobutylicum* has a very poor effect on misrepair via an error-prone pathway by UV.[11] Therefore, other methods have been carried out such as n-butanol tolerance in liquid culture[9] or n-butanol of solvent production: n-butanol resistant mutants SA-1[9] and strain 904 mutant[17] were obtained from *C. acetobutylicum*, respectively. And strain 77 mutant[18] was also isolated from *C. acetobutylicum*. OBT7 strain was clearly obtained from N1-4 strain by UV light and n-butanol tolerance on agar plate culture.

Growth on glucose fermentation by OBT7 mutant and *Clostridium accharoperbutylacetonicum* N1-4

OBT7 mutant produced 16.6 g of n-butanol, 1.48 g of ethanol, 4.02 g of acetone, 1.83 g of acetic acid, and 0.97 g of n-butyric acid per liter from 58.0 g (322 mM) of consumed glucose for 50 h (Fig. 1). H_2 and CO_2 were 197 mM and 644 mM, respectively (Table 1). After 3.5 h of lag phase, exponential growth phase continued for 27.9 h. Compared to N1-4 (Fig. 2), the log phase was long, considering glucose concentration was high. DWC of the cells was 1.55 mg per m*l* of the culture in the stationary phase. Maximum production of H_2 was 19.04 mM for 2 h (24.25~26.25 h) in the late log phase. And then H_2 production diminished suddenly.

N1-4 accumulated 11.65g of n-butanol, 2.63 g of acetone, 0.95 g ethanol, 1.54 g of acetic acid, and 0.23 g of n-butyric acid per liter from 39.41 g(219 mM) of consumed glucose for 37.5 h (Fig. 2). H_2

and CO_2 were 113 mM and 405 mM, respectively (Table 1). Lag phase was 3.5 h and log phase was 24 h. Dry weight of the cells (DWC) was 1.25 mg per m*l* of the culture in the stationary phase. Maximum production of H_2 was 16.2 mM for 2 h (24.5~26.5 h) in late log phase. The period of maximum H_2 production corresponding to the reducing start time of both acetic acid and n-butyric acid and concurrently, to the increasing time of n-butanol, ethanol, and acetone, indicating conversion of acids into solvents. These same results were also shown on fermentation by OBT7 mutant (Fig. 1). And then the H_2 production diminished suddenly, while n-butanol formation showed an increase, indicating reduction of H^+ into n-butanol.

On the other hand, the 904 mutant strain,[17] which is the strongest strain reported in production of acetone and butanol, produced 14.1 g of n-butanol, 6.6 g of acetone and ethanol, acetic acid, n-butyric acid per liter.

Compared to the parent N1-4 strain, OBT7 mu-

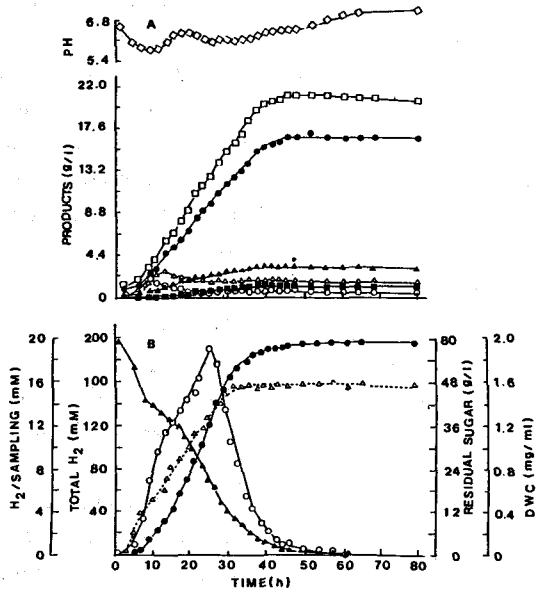

Fig. 1. Time course of products, cell growth, residual sugar, H_2 and pH on 609 g/*l* of glucose fermentation by OBT7 mutant at 30℃. A: ◇—◇, pH; □—□, total solvents; ●—●, n-butanol; ▲—▲, acetone; ■—■, ethanol; △—△, acetic acid; ○—○, n-butyric acid; B: ▲—▲, residual sugar; △---△, cell growth; ●—●, total H_2; ○—○, H_2 per sampling

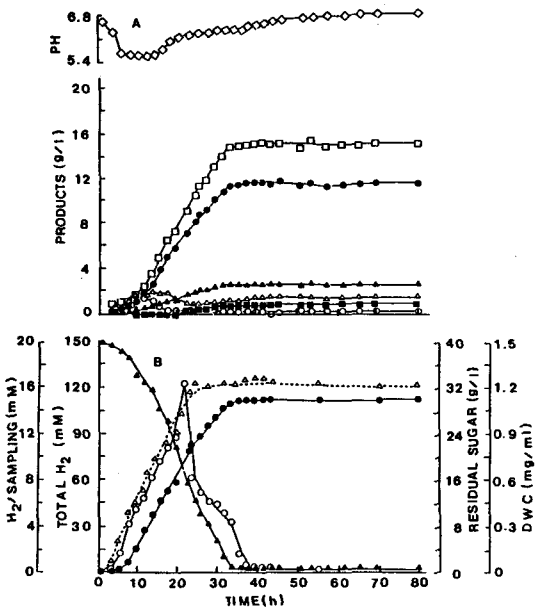

Fig. 2. Time course of products, cell growth, residual sugar, H_2 and pH on 40 g/*l* of glucose fermentation by *C. saccharoperbutylacetonicum* N1-4 ATCC 13564 at 30℃. A: ◇—◇, pH; □—□, total solvents; ●—●, n-butanol; ▲—▲, acetone; ■—■, ethanol; △—△, acetic acid; ○—○, n-butyric acid; B: ▲—▲, residual sugar; △---△, cell growth; ●—●, total H_2; ○—○, H_2 per sampling

Table 1. Carbon recovery, balance of available hydrogen, and oxidation-reduction balance on glucose fermentation by *C. saccharoperbutylacetonicum* N1-4 and OBT7 mutant

Strain	Glucose consumed (mM)	End products (mM)							Carbon recovery (%)	Hydrogen recovery (%)	OR balance
		n-Butanol	Acetone	Ethanol	Acetic acid	n-Butyric acid	H_2	CO_2			
N1-4	219	157	45.3	20.6	25.7	2.61	113	405	97.0	88.2	0.92
OBT7	322	227	69.2	12.8	15.0	3.97	197	644	94.7	84.9	0.98

tant produced 1.43 times of n-butanol, 1.52 times of acetone and consumed 1.47 times of glucose than that of N-1 strain. These results indicate that the translocation processes coupling to the chemical conversion of glucose were more active than that of N1-4, causing increase of glucose penetrated membrane. And in comparison of solvent productivity of 904 mutant, OBT7 mutant produced 17.7% more n-butanol and 64.2% less acetone than that of 904 mutant strain. And solvents yield of n-butanol and acetone were produced 31% higher and 6% higher than that of 904 strain. These results showed the posibility of industrial butanol and acetone production by OBT7 mutant.

Fermentation balance of OBT7 mutant and *C. saccharoperbutylacetonicum* N1-4

Fermentation balances of OBT7 mutant and N 1-4 strain is shown inTable 1. Carbon recovery was 94.7% and 97.0% for OBT7 mutant and N1-4, respectively. And oxidation-reduction balance was 0.98 and 0.92 for OBT7 mutant and N1-4, respectively. Also balance of available hydrogen is shown. These results show that the other products were not produced.

Influences of mannitol as the sole carbon source by OBT7 mutant and *C. saccharobutylacetonicum* N1-4

On mannitol fermentation by OBT7 mutant, both acetone and acetic acid were not produced, while n-butanol (9.09 g/*l*), ethanol (1.32 g/*l*) and n-butyric acid (3.14 g/*l*) increased by increasing the concentration of mannitol to 60 g of mannitol (Table 2). These results indicated that oxidized compounds coverted to reduced compounds. Compared to 60 g/*l* of glucose fermentation (Fig. 1), acetone and n-butanol were definitely diminished by 99.2% and 37.2 %, respectively. But n-butanol ratio and ethanol ratio to total solvents increased by 10.3% and 10.5%, respectively, totalling 20.9%, while the ratio of acetone to total solvents decreased by 21.3%. Especially at the 20 g/*l* of mannitol, the ratio of n-butanol to total solvents increased up to 94.8%, and acetone was not detected (Table 2). These results showed change of intersolvents in OBT7 mutant.

N1-4 strain produced 7.24 g of butanol, 1.07 g of ethanol, 0.42 g of acetone, and 0.46 g of acetic acid per liter on 40 g/*l* of mannitol fermentation. Compared to 40 g/*l* of glucose fermentation (Fig. 2), N1-4 produced less acetic acid and no n-butyric acid, indicating the acids were reutilized for reduced compounds. n-Butanol ratio and ethanol ratio to total solvents increased by 8.34% and 7.33%, respectively, totalling 15.7, while the acetone ratio decreased by 15.6%. These results showed the same trend as OBT7 mutant.

But the ratio of n-butanol to acetone was higher on mannitol fermentation than on glucose fermentation in the 2 strains: the ratios of n-butanol to acetone were 17.2 and no production of acetic acid for N1-4 strain and OBT7 mutant on 40 g/*l* and 60 g/*l* of mannitol fermentations, respectively (Table 2); on 40 g/*l* and 60 g/*l* of glucose fermentations, the ratios of n-butanol to acetone were 4.4 and 4.1 for N1-4 strains and OBT7 mutant, respectively (Fig. 1, 2). Compared to glucose fermentation, these results indicated that mannitol increased reduced compound formation such as acetone and by the two strains. It explains that an excess of 2 atoms

Table 2. Effect of mannitol of fermentation[a] by OBT7 mutant

Mannitol (g/*l*)	Products (g/*l*)					Butanol/ Acetone (ratio)	Ethanol/ Total solvents	Acetone/ Total solvents	n-Butanol/ Total solvents
	Ethanol	Acetone	n-Butanol	n-Butyric acid	Total solvents				
20	0.42	0.00	7.59	1.32	8.01	–	5.24	0.00	95.8
40	1.20	0.00	8.02	2.41	9.22	–	13.02	0.00	87.0
60	1.32	0.00	9.09	3.14	10.45	–	12.63	0.35	87.0
80	0.77	0.00	6.80	4.12	7.61	–	10.12	0.53	89.4

[a] Fermentation was done as described in fermentation process at 30℃

of hydrogen in mannitol molecule relates to reduction reaction causing increase of reduced compound formation by OBT7 mutant and N1-4 strain.

In acid formation on mannitol fermentation, compared to glucose fermentation, OBT7 mutant showed no acetic acid and increase of n-butyric acid

Table 3. Effect of temperature on glucose (70 g/l) fermentation[a] by OBT7 mutant

Temperature (℃)	Products (g/l)					Eluted gases (g/l)	H_2[b] (mM)	CO_2[b] (mM)	T-S (g/l)	CS (g/l)
	Et-OH	ACTN	ACOOH	Bu-OH	BCOOH					
20	0.62	1.62	0.58	9.58	0.40	10.00	324	385	11.8	31.5
25	0.70	1.95	0.83	9.45	0.17	12.30	388	403	12.1	32.4
30	0.87	4.00	1.00	16.5	1.31	27.50	979	794	21.4	60.8
35	0.68	4.72	1.24	11.2	0.73	29.40	1162	698	16.6	49.6
40	0.63	3.95	1.71	7.14	0.74	20.10	826	489	11.7	35.9

Bu-OH/ACTN	Et-OH/T-S (%)	ACTN/T-S (%)	Bu-OH/T-S (%)	T-S/CS (%)	Et-OH/CS (%)	ACTN/CS (%)	Bu-OH/CS (%)
5.92	5.25	13.7	81.1	37.5	1.97	5.14	30.4
4.85	5.79	16.1	78.1	37.4	2.16	6.02	29.2
4.14	4.06	18.7	77.3	35.2	1.43	6.58	27.2
2.38	4.09	28.4	67.5	33.5	1.37	9.52	22.6
1.81	5.38	33.7	60.9	32.6	1.75	11.0	19.9

[a] Fermentation was done as described in the text.

[b] Theoretical calculation of H_2 and CO_2 from the consumed sugar and the produced products. Et-OH, ethanol; ACTN, acetone; ACOOH, acetic acid; Bu-OH, n-butanol; BCOOH, n-butyric acid; CS, consumed sugar; T-S, total solvents.

Table 4. Effect of temperature on glucose (40 g/l) fermentation[a] by *C. saccharoperbutylacetonicum* N1-4 ATCC 13564

Temperature (℃)	Products (g/l)					Eluted gases (g/l)	H_2[b] (mM)	CO_2[b] (mM)	T-S (g/l)	CS (g/l)
	Et-OH	ACTN	ACOOH	Bu-OH	BCOOH					
20	0.02	1.06	1.21	5.40	0.77	5.80	266	233	6.48	19.7
25	0.52	2.13	0.67	10.9	0.34	18.3	436	455	13.6	36.5
30	0.42	2.22	0.73	9.55	0.22	14.8	665	474	12.2	34.7
35	0.20	4.46	1.43	8.46	0.39	21.4	944	550	13.1	39.4
40	0.32	5.20	2.18	8.22	0.75	22.0	547	463	13.7	38.9

Bu-OH/ACTN	Et-OH/T-S (%)	ACTN/T-S (%)	Bu-OH/T-S (%)	T-S/CS (%)	Et-OH/CS (%)	ACTN/CS (%)	Bu-OH/CS (%)
5.09	0.31	16.4	83.3	32.9	0.10	5.39	27.4
5.12	3.83	15.7	80.5	37.2	1.43	5.84	29.9
4.30	3.45	18.2	78.3	35.1	1.21	6.40	27.5
1.90	1.52	34.0	64.5	33.3	0.51	11.3	21.5
1.58	2.33	37.9	59.8	35.4	0.82	13.4	21.2

[a] Fermentation was done as described in fermentation process.

[b] Theoretical calculation of H_2 and CO_2 from the consumed sugar and the produced products. Et-OH, ethanol; ACTN, acetone; ACOOH, acetic acid; Bu-OH, n-butanol; BCOOH, n-butyric acid; CS, consumed sugar; T-S, total solvents.

(Table 2). These results showed that acid formation of OBT7 mutant differ from N1-4, which produced decreased acetic acid and no n-butyric acid as reported by Speakman.[19] It suggests that reduction reaction occurs more weakly in N1-4 than that of OBT 7 mutant by an excess hydrogen of mannitol molecule. On the other hand, on the view point of acid formations from terminal -OH groups,[19] these results suggest that the difference of acid production between glucose and mannitol fermentation may be caused by two pairs of adjacent -OH groups in mannitol molecule as comparison with one pair of the -OH group in glucose. This acid formation pattern of N1-4 also differed from the results of Johnson *et al.*,[20] who reported that acetic acid and n-butyric acid increased in a larger percentage of n-butyric acid on mannitol fermentation by *C. acetobutylicum.*

For ethanol mechanism, Johnson *et al.*[20] reported that the mechanism of *C. acetobutylicum* is independent of reduction reaction caused by the decrease of ethanol production on mannitol fermentation. But in this work, the ethanol mechanism depended on the reduction reaction caused by the increase of ethanol on, mannitol fermentation. The ethanol mechanism depended on reduction reaction is supported by control of electron flow utilizing CO gas on glucose fermentation.[21]

Effects of temperature on solvent production by OBT7 mutant and *C. saccharoperbutylacetonicum* N1-4

OBT7 mutant produced both maximum n-butanol (16.5 g/*l*) and maximum ethanol (0.9 g/*l*) at 30℃, and produced maximum acetone (4.7 g/*l*) at 35℃, on glucose fermentation (Table 3). The ratio of n-butanol to total solvent was decreased, but the ratio of acetone was increased, and glycolysis was increased, by increasing the culture temperature (Table 3). These results indicated that n-butanol yields showed a decrease, whereas acetone yields showed an increase in proportion of solvent production to consumed glucose, by increased temperatures.

N1-4 strain produced both maximum n-butanol (10.9 g/*l*) and maximum ethanol (0.5 g/*l*) at 25℃, and produced maximum acetone (5.2 g/*l*) at 40℃, on glucose fermentation (Table 4). The ratio of n-butanol and acetone to total solvent showed the same trend as OBT7 mutant. Also n-butanol yields and acetone yields from consumed sugar showed the same trend as OBT7 mutant (Table 4).

These results indicated that the optimum temperature for both butanol and ethanol production are appropriate at low temperatures range of 25～30℃ and for acetone production is suitable at a high temperatures range of 35～40℃, in the two strains.

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클로스트리디움 싸카로퍼부틸아세토니컴 N1-4株로부터 부타놀 다량생산株 OBT 돌연변이의 분리와 특성

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초록 : 1) OBT mutant는 *Clostridium saccharoperbutylacetonicum* ATCC 13564(N1-4株)로부터 UV light와 butanol tolerance에 의해 분리했다. 동 돌연변이株는 16.46 g/*l*(1.4배 증가)의 부타놀과 4.65 g/*l*(1.5배 증가)의 아세톤을 생산하고 전체 용매는 21.47 g/*l*를 생산했다. 이 결과는 n-butanol을 생산하는 clostridial bacteria에서 error-prone pathway를 통한 misrepair의 약한 효과가 UV light와 butanol tolerance에 의해서 극복되었다는 것을 제시했다. 2) glucose 발효에 비교해서 mannitol 발효에서 OBT mutant는 acetone과 acetic acid는 생산되지 않았다. 전체용매에 대한 n-butanol과 ethanol의 비는 각각 10.3%와 10.6%씩 증가되었고 전체적으로는 20.9% 증가된 반면, acetone의 비는 21.2%가 감소되었다. 또한 전체 용매에 대한 n-butanol의 최대비는 94.8%까지 증가하였다. 이들 결과는 산화합물(acetone, acetic acid, butyric acid)이 환원화합물(n-butanol, ethanol)로 전환된 것을 의미했다. 따라서 mannitol은 부산물인 산화합물을 제거하는데 사용할 수 있다.