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EFFECT OF SOME PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS ON ACETONE BUTANOL PRODUCTION BY *CLOSTRIDIUM ACETOBUTYLICUM*

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The effect of some fermentation conditions as well as the role of some additives on the production of acetone-butanol by a local strain of *C. acetobutylicum* were studied. Maximum solvent yield was obtained at pH 5.5, after 4 days incubation, using 10 ml inoculum, 43.25x10 cells and 24 h old culture. Addition of acetic acid and butyric acid separately and in mixture increased the solvent productivity by 10.4, 9.4 and 11.6%, respectively. Propionic acid at concentration of 20m mol produced the highest yield of solvents. On the other hyand butnaldehyde at lower concentration had no effect and at higher concentrations decreased the solvent yield sharply.

Key words: Acetone-butanol production, *C. acetobutylicum*, Additives.

Introduction

Acetone-butanol production by *Clostridium acetobutylicum* due to its economic importance has been studied intensively during the past few decades. The influence of pH of the fermentation medium has been recognized as key factor in determining the outcome of the process. Several studies reported that cultures maintained at high pH values produced mainly acids, while at lower pH the solvent production usually predominated (Jones and Woods 1986). However, the pH range over which solvent formation may occur appears to vary quite widely depending on the particular strain and the culture conditions used. *C. acetobutylicum* has been reported to produce good yield of solvents over a wide range of pH (4.5-6.0) (Geng and Park 1993). The same authors found that optimum pH for solvent production by the experimental organism was 5.5. This value was higher when compared with the optimum pH (4.3 to 4.5) for *C. acetobutylicum* DSM strains (Nishio *et al* 1983).

Acetic and butyric acids are produced during the acidogenic growth phase and induce the solvent formation in the second phase. They act as co-substrates and control the amount of solvents produced (Fond *et al* 1985). Also these acids are by their nature toxic to the cell. It has been suggested that the shift to solvent production in *C. acetobutylicum* is an adaptive response of the cell to inhibitory effects produced by acid end products (Bahl *et al* 1982). The shift to solvent production appears to act as a detoxification mechanism which allows the cell to avoid the inhibitory effects when acid end products reach toxic levels. The ratio of end metabolites depends upon acetic and butyric acid quantities produced during the fermentation. In contrast to acetic acid, which specifically increases acetone formation, butyric acid increases both acetone and butanol formation (El-Ammouri *et al* 1987).

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Materials and Methods

Microorganism and culture conditions. The bacterium used in this study was isolated from local soil sample taken from the rhizosphere of bean according to Weizmann's method and adopting successive heat shocking (Calam 1979).

Inocula were prepared by adding 0.2 ml of spore suspension to 10 ml of potato-glucose medium in anaerobic tubes with butyl bungs and aluminium crimps. Potato-glucose medium contains the following ingredients (g^{-1} l): wet potato mash 250; glucose 5; $(NH_4)_2SO_4$ 3; $CaCO_3$ 1.5 and cysteine hydrochloride 0.5. The pH was adjusted to 5.5 prior to autoclaving. The spore suspension was heat shocked at 100°C for 2 min. This culture was used to inoculate (5% v/v) 50 ml of potato-glucose medium in a 100 ml serum bottle. The culture was incubated overnight and then used as inoculum for the experiments. Anaerobic conditions were maintained by sweeping oxygen-free nitrogen gas across the surface of the culture according to the method of Hungate (1969). For fermentation, 150 ml serum bottles containing 100 ml of the fermentation medium of the following composition (g/l) were used: cane-sugar molasses 100; $(NH_4)_2SO_4$ 3; $CaCO_3$ 1.5; rice bran 5; corn 5; corn bran, 5. Cultures were continuously vented to prevent the build up of gas pressure. The bottles were incubated at 32°C. Fermentation conditions were however changed according to the objective of the particular experiment.

Analytical methods. Solvents (acetone and butanol) were determined using a gas chromotograph (Hewlett-Packard 5890) equipped with a flame ionization detector and an autolinear temperature programmer after the pH of the sample was decreased below 1.8 by adding 4N HCl. The column was packed with 6.6% carbowax 2.0M 80/120 Carbopack BAW. Nitrogen was used as the carrier gas. The gas chromatograph oven temperature was programmed from 120°C to 180°C. The temperatures of the injector and the detector were controlled at 180°C and 200°C, respectively (Geng and Park 1993). Reducing and non-reducing sugars of molasses were determined before and after acid hydrolysis whereas the total sugar content in the fermentation liquor was determined after hydrolysis according to the modified Schoorl's method (1929).

pH-relations. Aliquots of the basal medium were initially adjusted either with 1N solutions of HCl or NaOH to pH values ranging from 4.5 to to 7.5. All pH adjustments were carried out using PYE-UNICAM pH-meter.

Effect of the cultivation conditions. The incubation period. In this experiment solvent yields were determined at different time intervals ranging from 18-144 h.

Inoculum size. This experiment was carried out to study the effect of inoculating the fermentation medium with different inoculum sizes which ranged from 3 to 12 ml inoculum containing 43.25x10 cells m l^{-1} (using Haema cyto-meter MNK-400H).

Inoculum age. The effect of inoculum age on acetone-butanol production was studied by inoculating the fermentation medium with 10% inoculum of *C. acetobutylicum* at different ages.

Response to some additives. In the present series of experiments, the ability of the experimental organism to produce acetone-butanol was investigated when it was cultivated on the fermentation medium and fortified with various tested additives i.e. acetic acid (1-7 g l^{-1}), butyric acid (0.5-9 g l^{-1}) pyruvic acid (5-25 m mol), propionic acid (5-40 m mol) and butraldehyde (1-14 m mol).

Results and Discussion

pH-relations. The initial pH of the fermentation medium exerts a noticeable effect on the solvent yield. Maximum solvent production (16.27 g l^{-1}) and the highest value of sugar consumption (49.5 g l^{-1}) were obtained at pH 5.5 (Fig 1). The optimum pH for solvent production was similar to that for *C. acetobutylicum* IAM 19012 (Taya *et al* 1985) and was higher as compared to the optimum pH 4.3 to 4.5 for *C. acetobutylicum* ATCC 824 (Monot *et al* 1983, 1984) and *C. acetobutylicum* DSM strains (Nishio *et al* 1983).

Effect of the incubation period. The results of this investigation (Fig 2) showed that acetone-butanol production is time dependent. The highest solvent yield (18.56 g l^{-1}) was attained after 4 days of incubation. These results are in accordance with those of Quratulain *et al* (1995) who found that the optimum solvent yield was achieved after 4 days of incubation using molasses 60g l^{-1} (12%). In another study using synthetic medium containing glucose (62 g l^{-1}), the maximum output of solvents was achieved after only 30 h of incubation.

Effect of inoculum size. Results (Fig 3) showed that the total solvent yield increased with the increase of the inoculum size, reaching an optimum value at inoculum size of 43.25x10 cell per 10 ml inoculum. The inoculum size has been reported

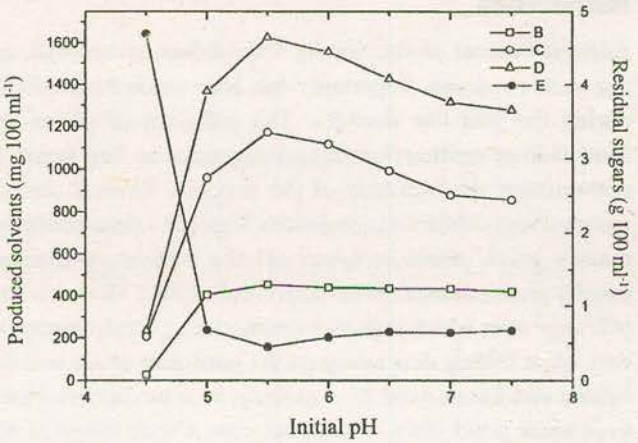

Fig. 1 Effect of the pH of the fermentation medium on acetone-butanol production by *Clostridium acetobutylicum*. (B, acetone; C, butanol; D, total solvents; E, residual sugar).

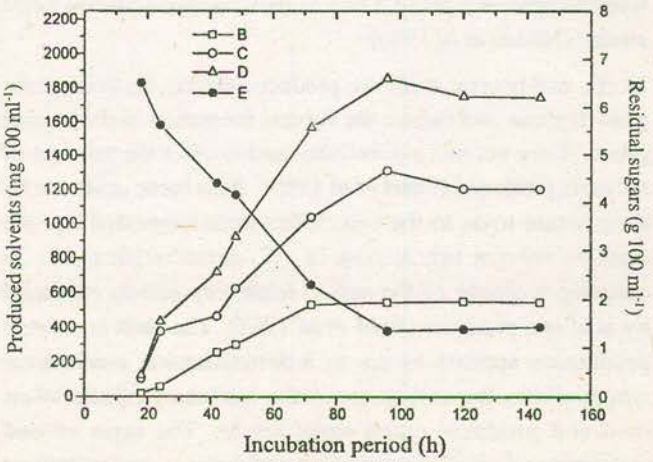

Fig. 2 Effect of the incubation period on acetone-butanol production by *C. acetobutylicum*. (B, acetone; C, butanol; D, total solvents; E, residual sugar).

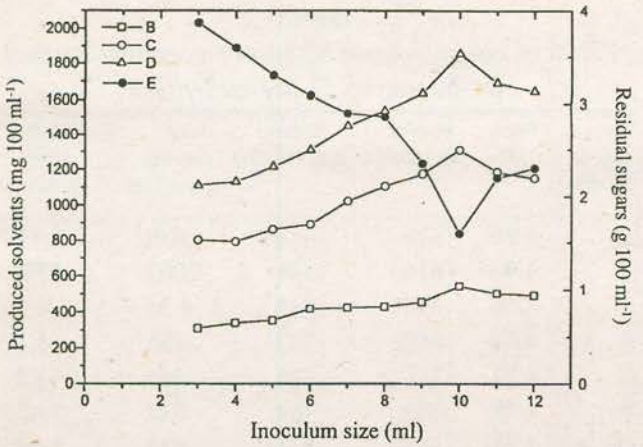

Fig. 3 Effect of inoculum size on acetone-butanol by *C. acetobutylicum.* (B, acetone; C, butanol; D, total solvents; E, residual sugar).

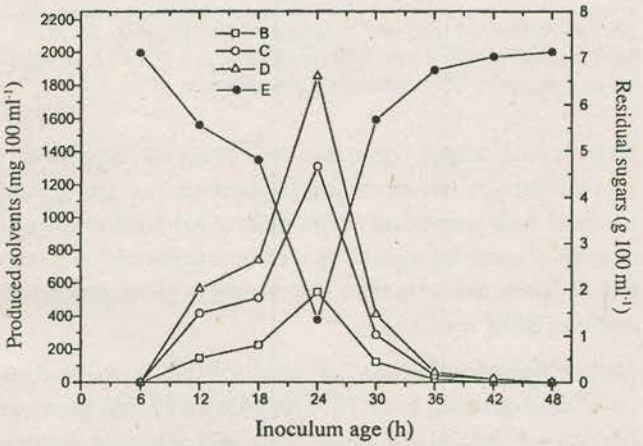

Fig. 4 Effect of inoculum age on acetone-butanol by *C. acetobutylicum.* (B, acetone; C, butanol; D, total solvents; E, residual sugar).

to be within the range of 5% (Welsh *et al* 1987) to 10% (v/v) (Rao and Mutharasan 1988; Larrayoz and Puigjaner 1987; Fond and Engasser 1986).

Effect of inoculum age. The data (Fig 4) showed that the optimum solvent yield (18.56 g l^{-1}) was obtained using 24 h old culture. On the other hand, sharp decrease in solvent output was recorded on using inoculum older than 24 h. In agreement with these results, the optimum age of inoculum was reported to be 24 h by many workers (Davies and Stephenson 1941; Holt *et al* 1984; Geng and Park 1993; Fond and Engasser 1986).

Response to some additives. A series of experiments were made using different levels of acetic and butyric acids separately and in mixture (Tables 1, 2, 3) to assess their effects on the solvent yield. The results revealed that the optimum concentrations of acetic and butyric acids were 2 and 0.5 g l^{-1} respectively when added separately and were 2 and 5 g l^{-1}, respectively in mixture. These concentrations

Table 1

Effect of acetic acid addition on acetone-butanol production by *C. acetobutylicum*

Acetic acid (g l^{-1})	Final pH	Acetone (mg 100ml^{-1})	Butanol (mg 100ml^{-1})	Total solvents (mg 100ml^{-1})	Residual sugars (g 100ml^{-1})
1	4.98	689	1552	2241	1.06
2	5.38	689	1631	2320	0.95
3	5.49	689	1593	2282	1.23
4	5.41	689	1433	2122	1.42
5	5.52	681	1355	2036	1.55
6	5.38	681	1276	1957	1.62
7	5.53	450	453	903	4.65
Control	4.92	616	1414	2030	1.15

Medium composition (g l^{-1}, wv^{-1}): molasses, 140; $(NH_4)_2SO_4$, 2; $CaCO_3$, 1; rice bran, 5; corn bran, 5; cysteine; hydrochloride, 0.7; at pH 5.5; incubation period, 4 days at 32°C; control, without acetic acid.

Table 2

Effect of butyric acid addition on acetone-butanol production by *C. acetobutylicum*

Butyric acid (g l^{-1})	Final pH	Acetone (mg 100ml^{-1})	Butanol (mg 100ml^{-1})	Total solvents (mg 100ml^{-1})	Residual sugars (g 100ml^{-1})
0.5	5.04	703	1671	2374	0.7
1	5.04	696	1672	2368	0.98
2	5.12	696	1572	2268	1.12
3	5.13	689	1375	2064	2.38
4	5.16	372	835	1207	4.62
5	5.34	225	604	829	5.28
6	5.21	152	510	662	5.74
7	5.29	106	489	595	6.02
8	5.25	34	386	420	6.58
9	5.30	34	176	210	6.83
Control	4.92	616	1414	2030	1.15

Medium composition (g l^{-1}, wv): molasses, 140; $(NH_4)_2SO_4$, 2; $CaCO_3$, 1; rice bran, 5; corn bran, 5; cysteine hydrochloride, 0.7; at pH 5.5; incubation period, 4 days at 32°C; control: without butyric acid.

afforded solvent yields of 35, 34.7, 35.4%g solvent per gram of sugar, respectively (compared with control 31.7%). The increase in productivity was 10.4, 9.4, 11.6% respectively. Our results agreed with those of Martin *et al* 1983, who found that the addition of acetic acid at concentration of 2 g l^{-1} to a culture of *C. acetobutylicum* with a solvent yield on glucose of 32% resulted in a yield of 34%. Addition of butyric acid (2 g l^{-1}) increases the production of all solvents to give an overall yield of 35%. The addition of both butyric and acetic acids (2 g l^{-1} each), gave a solvent yield of 34.7%. Datta and Zeikus (1985) reported that, the optimum initial concentrations were found to be about 2 g of acetic acid and

Table 3

Effect of addition of acetic and butyric acids on acetone-butanol production by *C. acetobutylicum*

Butyric acid ($g l^{-1}$)	Final pH	Acetone (mg $100ml^{-1}$)	Butanol (mg $100ml^{-1}$)	Total solvents (mg $100ml^{-1}$)	Residual sugars (g $100ml^{-1}$)
0.5+0.5	5.05	668	1554	2222	1.13
1+1	5.25	703	1552	2255	1.13
2+1	5.19	661	1614	2275	1.12
2+2	5.23	667	1648	2315	1.07
3+1.5	5.26	626	1231	1857	2.28
3+3	5.44	613	1556	2169	1.26
4+2	5.49	675	1615	2290	1.12
4+4	5.77	409	793	1202	4.68
5+2	5.50	681	1653	2334	0.98
5+5	5.51	576	864	1440	3.78
6+3	5.26	64	274	338	6.89
6+6	5.33	34	118	152	7.14
Control	4.92	616	1414	2030	1.15

Medium composition ($g l^{-1}$, wv^{-1}): molasses, 140; $(NH_4)_2 SO_4$, 2; $CaCO_3$, 1; rice bran, 5; corn bran, 5; cysteine; hydrochloride, 0.7; at pH 5.5; incubation period, 4 days at 32°C; control, without acetic and butyric acids.

Table 4

Effect of propionic acid addition on acetone-butanol production by *C. acetobutylicum*

Propionic acid (m mol)	Final pH	Acetone (mg $100ml^{-1}$)	Butanol (mg $100ml^{-1}$)	Total solvents (mg $100ml^{-1}$)	Residual sugars (g $100ml^{-1}$)
5	4.84	655	1516	2171	1.08
10	4.82	681	1594	2275	0.83
15	4.96	681	1613	2294	0.69
20	4.96	681	1632	2313	1.12
25	5.07	538	1279	1817	2.35
30	5.18	396	925	1821	3.9
35	5.22	350	799	1149	4.03
40	5.27	304	673	977	4.68
Control	4.92	616	1414	2030	1.15

Medium composition ($g^{-1} l$, wv^{-1}): molasses, 140; $(NH_4)_2 SO_4$, 2; $CaCO_3$, 1; rice bran, 5; corn bran, 5; cysteine hydrochloride, 0.7; at pH 5.5; incubation period, 4 days at 32°C; control, without propionic acid.

5 g of butyric acid per liter. This suggests that the initial concentration of the two acids strongly influence the final solvent yield.

Propionic acid addition. The results of this investigation (Table 4) showed that acetone-butanol production was enhanced by the addition of propionic acid up to 20 m mol. Huesemann and Papoutsakis (1988) reported that the addition of propionate enhanced the effect of undissociated butyric acid which correlates well with the initiation of solventogenesis. However, supplementation of the glucose minimal with propionate (20 m mol) at pH 5 led to the production of some *n*-propanol as well as acetone and *n*-butanol but in lesser amounts than the control without propionate addition (Holt *et al* 1984).

Table 5

Effect of butyraldehyde addition on acetone-butanol production by *C. acetobutylicum*

Butyraldehyde (m mol)	Final pH	Acetone (mg $100ml^{-1}$)	Butanol (mg $100ml^{-1}$)	Total solvents (mg $100ml^{-1}$)	Residual sugars (g $100ml^{-1}$)
1	4.96	616	1414	2030	1.15
2	4.90	616	1446	2062	1.06
3	4.66	390	446	8.36	4.2
4	4.68	369	387	756	4.7
6	4.72	218	468	686	4.92
8	4.75	203	469	672	5.02
10	4.77	161	411	572	5.18
12	4.78	119	273	392	5.45
14	4.68	112	213	325	5.71
Control	4.92	616	1414	2030	1.15

Medium composition ($g l^{-1}$, wv^{-1}): molasses, 140; $(NH_4)_2SO_4$, 2; $CaCO_3$, 1; rice bran, 5; corn bran, 5; cysteine hydrochloride, 0.7; at pH 5.5; incubation period, 4 days at 32°C; control: without butyraldehyde.

Butyraldehyde addition. The results (Table 5) revealed that addition of elevating levels of butyraldehyde slightly increased the butanol yield up to 2 m mol above which a sharp decrease in solvent yield occurred. Huesemann and Papoutsakis (1986) found that, the addition of 12 m mol butyraldehyde resulted in its conversion to butanol with a 70% yield (due to insolubility perhaps) but did not otherwise affect the culture characteristics as compared to the control culture.

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