APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1981, p. 889-893
0099-2240/81/040889-05$02.00/0
Vol. 41, No. 4

Comparison of Ethanol Production by Different *Zymomonas* Strains

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Received 4 August 1980/Accepted 15 January 1981

A comparison of the rates of growth and ethanol production by 11 different strains of *Zymomonas* revealed a wide range of characteristics, with some strains being more tolerant of high sugar or ethanol concentrations and high incubation temperatures than others. Some strains were unable to utilize sucrose; others produced large amounts of levan, and one strain grew well but produced no levan. One strain, CP4, was considerably better in all respects than most of the other strains and was chosen as a starting strain for genetic improvement of ethanol production.

Current interest in ethanol as a potential fuel has stimulated research on various aspects of the fermentation process. Different techniques for increasing productivity, such as continuous culture, cell recycle, and vacuum distillation, have been evaluated (2, 13), but another important consideration is the improvement of the fermenting organism to give maximum productivity.

One of the most promising ethanol-producing organisms is the bacterium *Zymomonas mobilis*, which is used to make palm wines and pulque and which also causes cider sickness (1, 7, 12, 14, 18). This bacterium can produce up to 1.9 mol of ethanol from each mole of glucose fermented, using a modification of the Entner-Doudoroff pathway (5, 6, 18). Recent reports from this laboratory have established that *Z. mobilis* can produce ethanol at a considerably higher specific rate than yeasts, and this has led to extremely high productivities in continuous culture with cell recycle (9, 15, 16).

Although *Z. mobilis* does, therefore, have considerable potential for ethanol production, it is likely that improved strains could be made by genetic manipulation. However, before attempting this, it is first necessary to select the best possible starting strain for ethanol production and genetic alteration. For this reason, the *Z. mobilis* strain which had been used for ethanol production studies in this laboratory was compared with various other *Zymomonas* strains isolated from several different locations.

MATERIALS AND METHODS

Bacterial strains. The various strains of *Zymomonas* used in this study are described in Table 1.

Media. All tests for growth and ethanol production were done in a rich medium containing 10 g of yeast extract (Oxoid), 2 g of KH_2PO_4, and 20, 100 or 200 g of glucose or sucrose made up to a total volume of 1 liter with distilled water. The KH_2PO_4 was added after autoclaving, and for solid medium, 15 g of agar (Difco) per liter was added.

Tests for ethanol production. Fresh log-phase cultures growing at 30°C in rich medium with 100 or 200 g of glucose or sucrose per liter were diluted 1:20 in fresh medium of the same composition. All tests were done at least twice in 20 ml of medium in test tubes (160 by 16 mm) incubated statically to minimize aeration. This was necessary to avoid the lowering of ethanol production rates and yields observed when cultures were aerated. Samples were periodically removed from the tubes and tested for ethanol in a Technicon Autoanalyser as previously described (9, 15). Rates of ethanol production were measured over a period of several hours in the middle of the exponential growth phase. Duplicate cultures always gave results within 3 g/liter of each other.

This method gave slower growth rates than could be obtained under optimum conditions in a fermentor (15, 16), but results were reproducible and, where tested, were confirmed in the fermentor.

Measurement of growth rate. Growth rates of cultures were measured by following the increase in optical density at 600 nm in a spectrophotometer. For cultures growing on sucrose, cells were first spun down and resuspended in fresh medium to remove the cloudy levan before the optical density was measured.

Estimation of levan production. To measure levan production, samples of cultures were first centrifuged at 7,000 rpm for 5 min to remove cells, but not levan, from the suspension, and the optical density of the remaining supernatant was then measured at 600 nm. Production of levan could also be detected visually since its presence made the medium cloudy.

To check that the cloudy substance was indeed levan, a portion of a culture of strain ZM4 grown in sucrose medium was centrifuged as described above, and the supernatant was treated with 75% ethanol to precipitate the cloudy substance. The brownish precipitate was collected by centrifugation at 7,000 rpm

for 15 min and was then resuspended in water. The suspension was hydrolyzed with 1 M HCl at 100°C for 15 min, after which the solution was completely clear. It was then assayed by high-pressure liquid chromatography and was found to contain only fructose. Therefore, the cloudy substance is a fructose polymer or levan (4).

RESULTS

Initially, 11 different strains of *Zymomonas* were grown in media containing 100 or 200 g of glucose or sucrose per liter at 30°C, to compare their rates of growth and ethanol production. In all cases it was found that ethanol production followed the same pattern as growth (i.e., fast growers produced ethanol faster). There was, however, considerable variation between the different strains, and data for strains growing on 100 g of substrate per liter are shown in Table 2. Strains such as CP4 and Z6 were observed to have a shorter lag period than others and also grew at a faster rate than other strains. *Z. mobilis* subsp. *pomaceae* strains S30.A, S30.2, 238, and ATCC 29192 were unable to use sucrose, although they mostly grew as well as other *Z. mobilis* strains on glucose (Table 2).

While growth on sucrose was being tested, it was observed that some strains produced more levan than others. Levan is a fructose polymer, characteristically produced by *Z. mobilis* growing on sucrose (4, 18). The presence of levan gives the medium an opaque, viscous appearance. Levan production by the various strains was assessed visually by comparing the growth media after 30 h of incubation, after removal of the cells by centrifugation. Most strains which grew on sucrose did produce levan, although strain Ag11 did not produce any detectable levan even if the concentration of sucrose in the medium was increased to 300 g/liter. The strain of *Z. mobilis* previously used for batch and continuous culture studies (strain ZM1 = ATCC 10988 [15, 16]) was found to produce more levan than most of the other strains. Production of this levan is wasteful in terms of sugar conversion to ethanol, so a strain which produced very little or no levan would be useful.

TABLE 1. *Zymomonas strains used*

Bacterium	Strain	Source	Reference/source
Z. mobilis	ATCC 10988	Fermenting *Agave* juice	10
	Ag11	Fermenting *Agave* juice	7
	3TH Delft	Fermenting *Arenga* sap	H. Derx; 15
	Z6	Fermenting *Elaeis* sap	19
	CP4	Fermenting sugarcane juice	O. Goncalves de Lima; 15
	B70	Infected British ale	3
	ZAbi	Beer	J. G. Carr
Z. mobilis subsp. *pomaceae*	ATCC 29192	Sick cider	15
	238	Cider	J. G. Carr
	S30.2	Apple pulp	J. G. Carr; 15
	S30.A	Cider	J. G. Carr

TABLE 2. *Growth and ethanol production by various Zymomonas strains growing on 100 g of glucose or sucrose per liter*

Strain	Glucose (100 g/liter)			Sucrose (100 g/liter)		
	Lag period (h)	Doubling time (h)	Ethanol (g/liter at 20 h)	Lag period (h)	Doubling time (h)	Ethanol (g/liter at 20 h)
ATCC 10988	2.8	3.1	25.7	2.5	3.4	23.2
Ag11	1.3	2.9	26.0	1.0	3.6	24.0
B70	1.5	3.9	22.0	1.8	3.8	25.1
CP4	1.2	2.8	33.7	1.5	3.0	25.0
3TH Delft	3.7	3.5	20.3	.0	3.7	23.6
ZAbi	2.4	4.2	14.3	1.8	3.8	25.0
Z6	1.4	2.4	27.5	1.0	3.1	29.2
ATCC 29192	2.4	3.2	31.0	>24		0
238	3.8	4.3	12.7	>24		0
S30.2	4.0	2.5	30.3	>24		0
S30.A	3.8	3.0	26.6	>24		0

Another consideration for selection of a *Zymomonas* strain suitable for ethanol production is its growth on solid media. This is important for selection of genetically altered colonies, where good growth on solid media is required. Therefore, the various strains were streaked on solid medium with 20 g of glucose per liter in petri dishes at 30°C, and the growth of colonies was measured.

Colony size after 2 to 3 days of incubation is shown in Table 3; the four strains ATCC 10988, CP4, Z6, and Ag11 grew faster than others on solid medium. Since these four strains also showed the best rate of ethanol production in liquid medium, they were chosen from the 11 initial strains for further study.

When the four strains were tested in liquid medium with 200 g of glucose per liter, strain CP4 was found to produce ethanol at a considerably faster rate than the other three strains (Table 4). The final ethanol concentration was also highest with this strain (Table 5), suggesting that CP4 may be less inhibited by ethanol than the other strains. This was confirmed by testing the growth of the four strains in liquid medium with 20 g of glucose and up to 80 g of added ethanol per liter. All four strains grew with 60 g of ethanol per liter, but only strain CP4 could grow in the medium with 80 g of ethanol per liter.

On 200 g of sucrose per liter at 30°C, strains ATCC 10988 and CP4 gave similar results, both producing ethanol faster than strains Ag11 and Z6 (Table 4).

When the four strains were grown at higher temperatures, all were found to grow and produce ethanol more slowly than at 30°C, and this was also reflected in their final ethanol concentrations (Table 5). However, once again strain CP4 appeared to be the most promising strain overall. It is interesting to note that growth on sucrose was less affected by increased temperature than growth on glucose. When levan production was measured at these higher temperatures, it was observed that the amount of levan was dramatically decreased as the temperature of incubation increased, with very little levan production occurring at 37°C (Table 6).

When the growth rates, rates of ethanol production, ethanol yields, levan production, and colony growth were all considered together, it was clear that strain CP4 was the most suitable starting strain for genetic improvement of ethanol production. This conclusion was further confirmed by a comparison of ATCC 10988 and CP4 in liquid medium with 300 g of glucose per liter. The maximum rate of ethanol production by strain ATCC 10988 under these conditions was 1.26 g/liter per h, compared with a rate of 2.17 g/liter per h for strain CP4 (measured during the exponential growth phase in static test tubes of rich medium at 30°C).

Strain CP4 has also been compared with strain ATCC 10988 in batch and continuous culture in a 1-liter fermentor, and the results obtained in this system have confirmed that CP4 is also superior to ATCC 10988 under these conditions (8).

TABLE 3. *Growth of Zymomonas strains on solid medium with 20 g of glucose per liter at 30°C*

Strain	Colony size (mm) after 72 h
ATCC 10988	2
Ag11	2
B70	1
CP4	3
3TH Delft	1.5
ZAbi	1
Z6	3
ATCC 29192	1.5
238	1
S30.2	1
S30.A	1

TABLE 4. *Ethanol production by Z. mobilis strains growing on 200 g of glucose or sucrose per liter*

Strain	Rate of ethanol production[a] (g/liter per h) from:	
	Glucose (200 g/liter)	Sucrose (200 g/liter)
ATCC 10988	1.43	1.22
Ag11	1.40	1.00
CP4	2.00	1.21
Z6	1.25	0.80

[a] Rates of ethanol production were measured during the exponential growth phase of the cultures in test tubes containing rich medium with 200 g of glucose or sucrose per liter at 30°C.

TABLE 5. *Final ethanol concentrations produced by Z. mobilis strains at different temperatures from 200 g of glucose or sucrose per liter*

Strain	Final ethanol concn (g/liter)					
	30°C		37°C		42°C	
	Glucose	Sucrose	Glucose	Sucrose	Glucose	Sucrose
ATCC 10988	60	56	40	45	0	28
Ag1	55	39	0	20	0	8
CP4	81	56	52	38	30	28
Z6	77	51	0	41	0	19

TABLE 6. *Levan production by Z. mobilis strains after 48-h growth on 100 g of sucrose per liter at different temperatures*

Strain	Levan produced (OD_{600})[a] at:			
	30°C	34°C	37°C	42°C
ATCC 10988	0.59	0.30	0.03	0.00
Ag11	0.00	0.00	0.00	0.00
CP4	0.48	0.38	0.04	0.00
Z6	0.82	0.60	0.14	0.00

[a] OD_{600}, Optical density at 600 nm.

DISCUSSION

For industrial ethanol production, several properties of the fermenting organism are very important in order to minimize the costs involved. The more concentrated the ethanol is in the residual medium, the cheaper it is to distill off (2, 13). Thus, the organism should be able to grow in high sugar concentrations and have a high ethanol tolerance. It should also produce ethanol at as fast a rate as possible, and if vacuum distillation is used, then growth at as high a temperature as possible reduces the amount of vacuum needed to distill off the ethanol (2, 13). A flocculant strain may also be required, depending on the method of cell recycle.

Although initial studies in this laboratory with *Z. mobilis* grown in batch and continuous culture clearly demonstrated its potential for ethanol production (9, 15, 16), only one strain was used. However, from the data presented here, it is apparent that there is considerable variation between different *Zymomonas* strains, with certain strains being much better than others for ethanol production. Obviously, if any sucrose-containing substrates are to be considered (such as molasses, sugarcane juice, or sugar beet), then strains of *Z. mobilis* subsp. *pomaceae* are inappropriate.

Of the *Z. mobilis* strains which grew on both glucose and sucrose, four were markedly better than the others with respect to growth rate, ethanol productivity, and growth on solid media. When these four strains were compared in detail, it was found that strain Ag11 was generally slower and gave lower yields, even though it did not produce any levan. Strain Z6 was also slower on high sugar concentration and produced large amounts of levan from sucrose. These two strains were much more sensitive to temperature than the other two strains.

When the two remaining strains, ATCC 10988 and CP4, were compared, it was immediately apparent that under all conditions of growth on glucose strain CP4 was superior to ATCC 10988. On sucrose media, growth and ethanol production by strains ATCC 10988 and CP4 were very similar, although CP4 produced slightly less levan. Three other *Z. mobilis* strains which, like CP4, were isolated from sugar cane juice behaved in a manner very similar to strain CP4 (data not shown).

For these reasons, strain CP4 was chosen as the most promising strain for ethanol production. This strain is also suitable for further improvement using genetic techniques, since it grows well on solid media and can accept and transfer several conjugable plasmids (17). Also, it is amenable to mutagenesis, and studies are currently under way in this laboratory to isolate mutants of CP4 which tolerate higher levels of ethanol and can use a wider range of substrates.

ACKNOWLEDGMENTS

J. G. Carr, J. DeLey, and O. Goncalves de Lima are thanked for kindly providing the *Zymomonas* strains used. R. J. Pagan is thanked for assaying the levan.

Support for part of this research was provided under the National Energy Research, Development and Demonstration Program administered by the Commonwealth Department of National Development and Energy.

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