0273-2289/94/4546—0283$03.40

Effect of Media Supplementation on Ethanol Production by Simultaneous Saccharification and Fermentation Process

I. BALLESTEROS, J. M. OLIVA,
J. C. CARRASCO, AND M. BALLESTEROS*

Div. Biomasa. IER-CIEMAT,
Avda. Complutense, 22 28040 Madrid, Spain

ABSTRACT

In this study, fermentation tests on different initial glucose concentrations, ranging from 100 to 200 g/L, were conducted to identify the ethanol tolerance of *Kluyveromyces marxianus* EMS-26 strain at 42°C. Lipid and nutrient additions to the fermentation medium were made in order to improve the ethanol production at 42°C.

The results obtained showed that the addition of unsaturated fatty acids and sterols to the SSF media reduced hydrolysis yields and ethanol production as compared to tests carried out on controls without lipid supplementation. These results seem to indicate that cellulases performed better when no lipid supplementation was used. The decrease in the saccharification in the presence of 0.5% Tween 80 (10% decrease in the filter paper activity) seems to be closely related to the denaturation of cellulase complex rather than lipids interference with cellulase binding.

Index Entries: Cellulose; simultaneous saccharification and fermentation (SSF); thermotolerant yeasts; ethanol tolerance; lipid and nutrient supplementation.

INTRODUCTION

The process of simultaneous saccharification and fermentation (SSF) for cellulose conversion into ethanol employs fermentative microorganisms in combination with the cellulase enzyme to minimize accumulation

*Author to whom all correspondence and reprint requests should be addressed.

of sugars in the fermenter. One problem associated with the SSF process is the different temperature optima for the action of enzymes and yeasts. Since the optimum saccharification temperature is close to 45–50°C, the use of thermotolerant yeast strains would be desirable in applying the coupled SSF process for cellulose conversion to ethanol.

There are a number of references in the literature describing yeasts that have the ability to grow at temperatures above 40°C (*1,2*), but there are few reports of high-yield ethanolic fermentations mediated by such organisms. Several authors (*3–5*) have identified the species belonging to the *Kluyveromyces* genera as the most thermotolerant yeasts able to grow and ferment glucose effectively at high temperatures, above 45°C. Despite its good thermotolerance, *Kluyveromyces* sp seem to have a low ethanol tolerance at the high temperatures, as indicated by the increased amount of residual sugars in the medium in the higher temperatures tested.

The ethanol adversely affects cellular physiology, resulting in reduced sugar consumption rates and overall decrease in metabolic rates (*6,7*). There is considerable evidence indicating that the cellular membrane composition is an important aspect of ethanol tolerance (*8,9*). The damage caused by ethanol to the cell membrane results in altered membrane organization and permeability. These studies have shown that the incorporation of unsaturated fatty acids, sterols, or both into the cellular membrane helps to alleviate ethanol inhibition. The addition of these components to the fermentation medium results in an overall increase in membrane fluidity (*10*), which overcomes the contrary effects attributed to ethyl alcohol. Several laboratories have also reported improvements in alcoholic fermentation and final ethanol concentration by broth supplementation with some additives, such as lipids, proteins, and vitamins (*11–14*). Supplementation with peptone, yeast extract, magnesium, or potassium salts also has a significative and positive effect on overall fermentation rates (*15,16*), indicating that nutrient limitation may play a primary and important role in the ethanol production and tolerance mechanism of yeasts.

In this study, the effects of broth supplementation with unsaturated fatty acids, sterols, and nutritional components on the production of ethanol from glucose by the simultaneous saccharification and fermentation process using *Kluyveromyces marxianus* are evaluated.

MATERIALS AND METHODS

Organisms and Growth Conditions

Kluyveromyces marxianus EMS-26, a thermotolerant mutant obtained in our laboratories from *K. marxianus* LG after treatment with ethylmethanosulfonate (*17*), has been used in this work. Active cultures for inoculation were prepared by growing the organisms on a rotary shaker (Braun Certo-

mat H) at 180 rpm for 16 h at 42°C, in a basal medium containing, g/L: yeast extract (Difco), 2.5; peptone (Oxoid), 5.0; NH_4Cl, 2; KH_2PO_4, 1; $MgSO_4$, 0.3; and glucose, 50.

Fermentation and SSF Assays

Glucose fermentation assays at 30 and 42°C were carried out in 100-mL Erlenmeyer flasks, each containing 50 mL of fermentation medium with initial glucose concentrations of 100, 150, and 200 g/L. Flasks were coupled with rubber stoppers with fermentative tubes filled with 2.5 mL of 50% H_2SO_4 to prevent ethanol evaporation. The flasks were inoculated with 10% (v/v) of yeast cultures. Sigmacell-50 (10% w/v) was used as substrate for SSF experiments.

The cellulase used was kindly provided by J. Pourquie from Institute Français du Petrole. Cellulase solutions were filtered-sterilized, and a cellulase enzyme loading of 15 PFU/g substrate was employed. It was obtained by ultrafiltration of a culture of the mutant *Trichoderma reesei* CL-847 followed by freeze-drying of the liquid fraction containing the enzyme. This fraction exhibited final enzymatic activities of 0.65 and 0.8 IU/mg of protein for filter paper and β-glucosidase, respectively.

A lipid mixture of unsaturated fatty acids and sterols was added to the media. The stock solution of lipid and sterols was prepared by dissolving ergosterol in ethanol and then adding Tween 80. This mixture was flushed with nitrogen. The final concentration in the fermentation media was 30 mg/L ergosterol and 5 g/L Tween 80.

Additional nutrients were added to the basal medium. This broth consisted of (g/L): yeast extract, 10; peptone, 10; NH_4Cl, 2; $MgSO_4\,7H_2O$, 1.7; KH_2PO_4, 9; CaCl, 0.3; $(NH_4)_2SO_4$, 7.5; NaCl, 1; $FeCl\,6H_2O$, 0.01; citric acid, 1; and glucose 200.

Analytical Procedures

Viability of yeast population was measured by cells staining with methylene blue (fermentations on glucose) and by plate counting (SSF experiments). A proportion (1.0 mL) of undiluted suspension was stained with methylene blue solution (0.01% w/v) for 5 min. Samples were appropriately diluted, wet preparations were examined under the microscope, and cells were scored for viability. Live cells remained colorless. Cell viability of the yeasts in SSF tests was controlled by streaking the samples on agar plates containing growing medium and checking for cell growth after 3 d of incubation at 30°C. Glucose and cellobiose were determined by HPLC using a Hewlett-Packard apparatus equipped with an RI detector, a column of Aminex HPX87 P at 85°C, and an eluant (water) flow rate of 0.6 mL/min. Ethanol was measured by GLC, utilizing a KONIK 2000 C Series apparatus with FI detector and a column of Carbowax 20*M* (2*M* × 1/8 in.) at 95°C. Injector and detector temperature: 125°C. Residual cellulose was estimated as described by Spindler et al. (*18*).

RESULTS

In order to study the ethanol tolerance of *K. marxianus* EMS-26 at temperatures close to those usually employed in SSF process, a preliminary study of the ethanol effects on fermentation parameters was carried out employing different initial glucose concentrations at 42°C. Analogous tests at 30°C were also made as a control. Effects of broth supplementation have also been studied.

Effect of Ethanol and Initial Sugar Concentration

A study on the effects of the presence of ethanol and of the initial glucose concentration on fermentation parameters of *K. marxianus* EMS-26 was performed on basal medium at 30 and 42°C. The data of these tests are summarized in Table 1, in which fermentation time, ethanol yield (Y, g ethanol/g glucose utilized x 100), and efficiency of substrate utilization (E, g glucose utilized/g initial glucose x 100) are listed. The fermentation time, Y and E values, were calculated at both temperatures from data where peak ethanol concentrations were recorded.

As observed in the cited table, similar ethanol yields were obtained when 100 and 150 g/L initial sugar concentration media were fermented at 30°C. However, fermentation time and ethanol production increased at 150 g/L initial glucose concentration. The glucose was completely metabolized at these fermentation conditions.

Two hundred grams per liter of initial glucose fermentations at 30°C showed a drastic increase in fermentation time and a relatively small increase in ethanol production (9.8%), although ethanol yield was not different from that obtained with lower initial sugar concentrations. The decrease in the efficiency of substrate utilization showed the inability of the microorganisms to ferment the glucose in the media completely. This fact, on the other hand, does not seem to be related to the initial osmotic pressure in the media, since the initial yeast growth and ethanol production rates were not significantly different in all cases studied. Accordingly, the increase of the fermentation time when 200 g/L initial glucose concentrations were used seems to indicate that the lower efficiency of substrate utilization was the result of alcohol intolerance rather than osmotic sensitivity. Moreover, this fact is also supported by results of tests at 42°C, which were similar to those obtained at 30°C for initial glucose concentration of 100 and 150 g/L, showing the good thermotolerance of the strain used. However, at the highest sugar concentration assayed, ethanol yield and substrate utilization efficiency were lower at 42°C than in 30°C fermentation assays. The decrease in ethanol production (20%) and the shorter fermentation time may be attributed to an inhibitory effect of ethanol that becomes more severe with increasing temperature.

Table 1
Effect of Initial Glucose Concentration on Fermentation Parameters at 30 and 42°C

Temperature, °C	Initial glucose, g/L	Fermentation time, h		Maximum ethanol concentration, g/L		Y[a] %		E[b] %	
		Basal media	Media supplemented with unsaturated fatty acids and sterols	Basal media	Media supplemented with unsaturated fatty acids and sterols	Basal media	Media supplemented with unsaturated fatty acids and sterols	Basal media	Media supplemented with unsaturated fatty acids and sterols
30	100	17	18	38.5	38.5	38.5	38.5	100	100
	150	25	24	58.3	56.3	38.9	37.5	99.5	100
	200	40	48	64.0	67.6	30.3	38.6	79.4	87.5
42	100	18	17	38.7	39.7	38.7	39.7	99.7	100
	150	24	25	56.6	57.3	37.7	38.2	100	100
	200	30	48	50.9	68.2	36.2	40.1	70.2	85.1

[a] Y% = g ethanol/g gluose utilized × 100.
[b] E% = g glucose utilized/g initial glucose × 100.

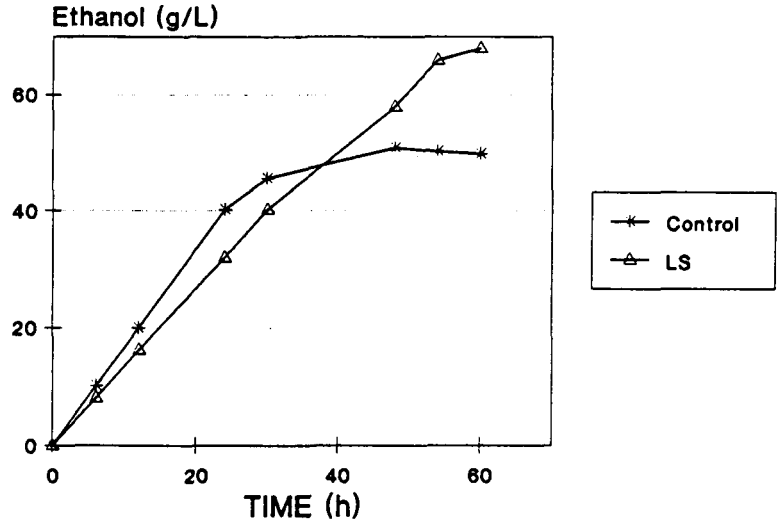

Fig. 1. Kinetics of ethanol in 200 g/L glucose fermentations without (control) and with lipid supplementation (LS) at 42°C.

Effect of Lipid Supplementation (LS)

In order to try to improve the ethanol production at 42°C, fermentations at this temperature were carried out in basal medium supplemented with unsaturated acids and sterols (Table 1). Analogous tests at 30°C were also made as a control.

Except with 200 g/L initial glucose concentration, no differences in fermentation parameters were observed when lipid supplementation was used. The addition of the lipids mixture in the experiments at 200 g/L initial glucose resulted in an increase in ethanol production and in the efficiency of substrate utilization, as compared to those obtained on basal medium.

Figure 1 presents the kinetics of ethanol production in a 200 g/L glucose medium with and without lipid supplementation at 42°C. The lipid mixture resulted in an increase in the maximum ethanol concentration, and contrary to expectations, lower product formation rates than in unsupplemented experiments were found. Two hundred grams per liter of initial glucose concentrations were used in further experiments to test the ethanol tolerance of yeast.

Effect of Lipid Supplementation on the Maximum Growth Rate (μm) and the Final Biomass Concentration (Xm)

It has been shown previously (*6,7*) that ethanol is inhibitory to cell growth and fermentation, and causes reduced cell viability. This fact is further accentuated at higher temperatures (*19*). To assess the effect of

Table 2
Effect of Media Composition
on Growth Parameters at 30 and 42°C

	Temperature °C	Control	LS
μm	30	0.21	0.15
(h^{-1})	42	0.10	0.07
Xm	30	6.0	5.0
(g/L)	42	3.6	3.7

Control: Fermentations on basal medium.
LS: Fermentations on lipid-supplemented medium.

lipid supplementation on growth parameters of the yeast employed in this work, the variation of the specific growth rate (μm) and the maximal biomass production (Xm) were measured in 200 g/L initial glucose concentration fermentations at 30 and 42°C.

As can be seen in Table 2, the cells grown on supplemented media presented slower growth rates than those obtained from basal media for both temperatures assayed. Cellular concentration (Xm) seems also to be influenced by media composition, according to the results obtained at 30°C. Nevertheless, at the higher temperature, lipid-supplemented cultures showed a slight decrease in Xm (26%) when compared to those obtained on basal medium (42%).

Effect of Lipid Supplementation on Cell Viability

When cultivated at 30°C, cell populations enriched with fatty acids remained viable to a similar extent to those cultivated on basal medium (Fig. 2). However, populations enriched with unsaturated fatty acids and sterols were more resistant to ethanol when fermentations were carried out at 42°C. Populations of cells grown in supplemented media at a higher temperature retained viability similarly to those cultivated at 30°C.

Effect of Nutrient Supplementation (NS)

Several authors (*13,19*), have reported that the factor limiting high yields of ethanol by brewing yeasts is a nutritional deficiency rather than ethanol toxicity. It was shown that increased ethanol production could be achieved at higher temperatures when additional nutrients were added to the medium. Such additives include not only lipids, but also proteins and vitamins. According to this, and in order to try to improve the results of the fermentation studied, tests of 200 g/L at 42°C were carried out on a basal medium supplemented with increased amounts of nutrients as defined in Material and Methods. It was determined that no effect was

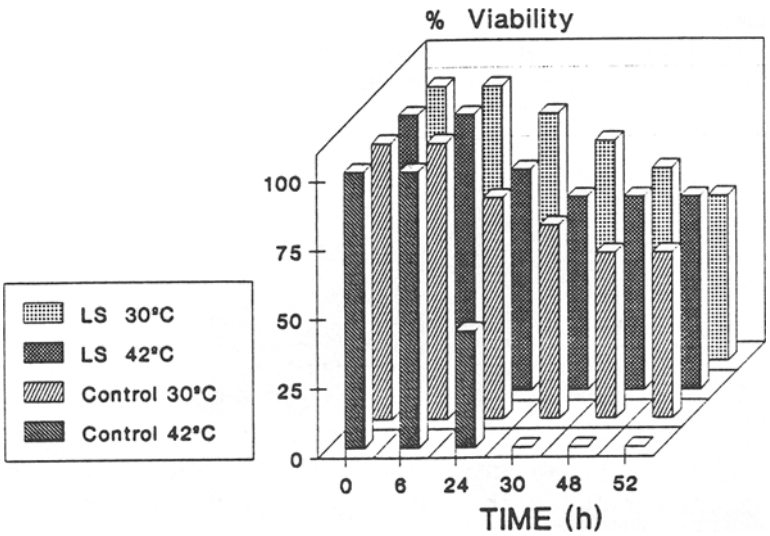

Fig. 2. Cell viability (%) of populations of *Kluyveromyces marxianus* EMS-26 on basal medium (control) and unsaturated fatty acids and sterols-supplemented medium (LS) at 30 and 42°C.

observed with increasing concentration of these nutrients as compared to those obtained with basal medium (data not shown).

Effect of Media Composition on SSF Process

Kluyveromyces marxianus EMS-26 was run in 50-mL SSF cultures at a substrate loading of 10% Solka-floc and a cellulase concentration of 15 FPU/g substrate at 42°C, in the following broth: control (basal medium), lipid supplemented (LS) (basal medium + unsaturated fatty acids and sterols), nutrient supplemented (NS) (basal medium + additional nutrients), and lipids- and nutrients-supplemented medium (LNS) (basal medium + lipid + nutrients). Figure 3 presents ethanol production data for these experiments.

There was no difference found in the extent of ethanol production between control and nutrient-supplemented broth. In distinction from the results obtained on glucose media, a lower ethanol concentration was obtained when unsaturated fatty acids and sterols were added to the SSF medium.

Cellulose degradation is another parameter measured in SSF that is related to the ethanol production. Figure 4 illustrates the percentage of cellulose hydrolyzed, and equivalent cellulose conversions, based on the measured ethanol concentration, after 6 d of SSF, for the different media assayed. Higher hydrolysis yields were obtained in SSF tests on control and nutrient-supplementated media as compared to those supplemented with unsaturated fatty acids and sterols. These results seem to indicate that cellulases performed better when no lipid supplementation was used.

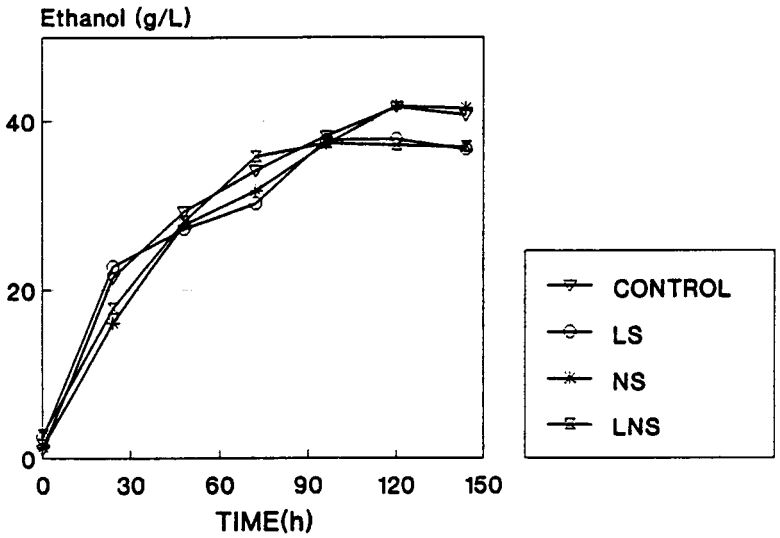

Fig. 3. Ethanol production of SSF process at 42°C in different broth composition (10% Sigmacell-50, cellulase loading 15 FPU/g substrate). Control: basal medium; LS: basal medium + lipid supplementation; NS: basal medium + additional nutrient; and LNS: basal medium + lipids + nutrients.

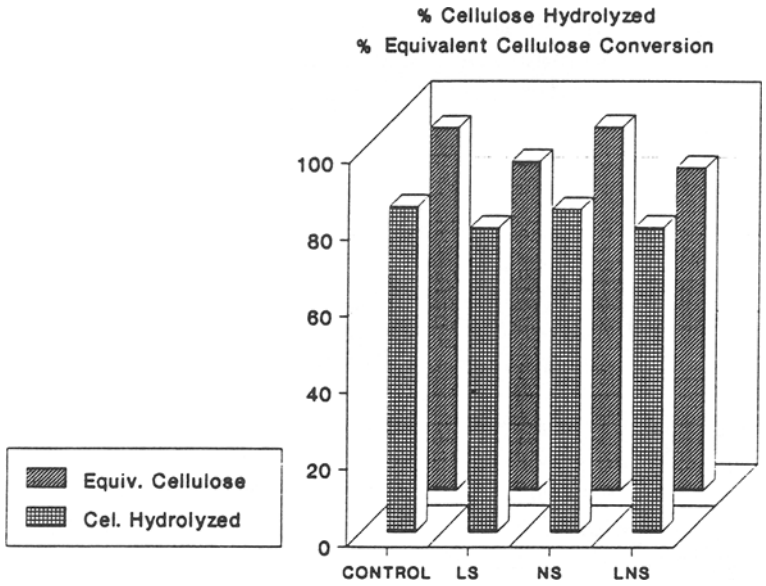

Fig. 4. Percentage of cellulose hydrolyzed and equivalent cellulose conversion from 6 d of SSF tests of Sigmacell-50 (10%) and cellulase loading 15 FPU/g substrate at 42°C. Control: basal medium; LS: basal medium + lipid supplementation; NS: basal medium + additional nutrient; and LNS: basal medium + lipids + nutrients.

In all cases tested, cultures maintained very low sugar concentration in the medium over a 5-d time period (lower than 0.5 g/L) (data not shown). From here on, residual sugars increased, but in all cases, small glucose concentrations, below 4 g/L, were achieved.

DISCUSSION

Kluyveromyces marxianus has been identified by several authors (*1–3*) as one of most thermotolerant yeasts able to produce ethanol from glucose between 37 and 45°C. In a previous work (*17*), a *Kluyveromyces marxianus* thermotolerant mutant strain (EMS-26) was selected because of its ability to carry out the simultaneous saccharification and fermentation of cellulose into ethanol. Despite its good thermotolerance, *K. marxianus* EMS-26 appears to have low ethanol tolerance at higher temperatures as indicated by the increased amount of residual sugars in the SSF tests at 45°C.

It has been documented (*11–14,20*) that nutrient supplementation can improve the fermentation of glucose at higher temperatures. Hayashida and Ohta (*8*) reported that yeasts acquired enhanced ethanol tolerance when grown in the presence of fungal mycelium extract containing unsaturated fatty acids.

In this work, the effect of nutrients and lipids addition on the production of ethanol from glucose and cellulose by *K. marxianus* EMS-26 was evaluated. Results obtained on glucose media at 42°C showed enhanced ethanol production and fermentation rates by the addition of a mixture of Tween 80 and ergosterol to the fermentation broth. This supplementation negatively affected the cellular biomass concentration (as can be seen in Table 2), but nevertheless, in the presence of the lipid mixture, the cells remained viable to a higher extent than those cultivated on an unsupplemented medium. It could be so concluded that the lipid supplementation seems to have a protective effect on the maintenance of cell viability at higher temperatures, but not on the growth of the strain.

The results obtained in fermentation tests with nutrients supplementation showed that no effect was achieved by increasing the concentrations of peptone and yeast extract, which may be related to the fact that enough nitrogen and vitamins were already present in the medium. These results suggest that the nutritional deficiencies were not responsible for the low cell yield, so basal broth was employed in further SSF studies.

The slight positive effects produced by the lipid supplementation obtained on glucose fermentation was not observed in SSF tests. Moreover, better performances were observed without lipid supplementation. The low residual sugar (glucose + cellobiose) concentrations in the medium of the SSF tests indicated a good performance of the thermotolerant cellobiose fermenting yeast *K. marxianus* EMS-26 both with and without

lipid supplementation. These data would suggest that the enzymatic hydrolysis step is affected by unsaturated fatty acids and sterols. This can also be supported by the fact that filter paper activity of cellulase complex was decreased by 10% when the SSF media were supplemented with unsaturated fatty acids and sterols.

There are some references in the literature (*21,22*) on the effect of surfactants on enzymatic saccharification of cellulose. Castanon and Wilke (*21*) have reported that the adsorption of filter paper activity on newspaper is hindered by 0.1% Tween 80, and that this difficulty of adsorption of enzyme prevents the immobilization of enzyme on the surface of substrate, resulting in an enhancement of saccharification of cellulose. The results obtained in this work, using 0.5% Tween, are not in keeping with those obtained by Castanon and Wilke.

Ooshima and coworkers (*22*) have reported that surfactants might have abilities as an accelerator and an inhibitor of the saccharification. At low concentrations (0.05% Tween 20), nonionic surfactants can act as an accelerator of saccharification, but the saccharification tends to be disturbed by denaturation of enzyme as the concentration of the surfactants become higher. In this context, the Tween 80 concentrations cited in the literature (*10*) as suitable to achieve a protective effect on cell viability are between 0.2 and 0.5%, several times higher than those employed by Ooshima et al. This could explain the decrease in the saccharification in the presence of 0.5% Tween 80 observed in this work, which could be related to the denaturation of the enzyme by the surfactant rather than lipids interference with cellulase binding.

According to the results shown in this work, it can be concluded that the lipid addition, at the concentrations usually reported in the literature to achieve enhanced ethanol tolerance and production by yeasts, has a negative effect on the ethanol production from SSF process by *K. marxianus* EMS-26 in the conditions studied in this work. Therefore, it should be of interest to conduct a further investigation of the suitability of such addition to improve the SSF process.

REFERENCES

1. Sczodrak, J. and Targonski, Z. (1988), *Biotech. and Bioeng.* **XX,** 300–303.
2. Hacking, A. J., Taylor, I. W. F., and Hanas, C. M. (1984), *Appl. Microbiol. Biotechnol.* **19,** 361–363.
3. Hughes, D. B., Tudroszen, N. J., and Moye, C. J. (1984), *Biotechnol. Lett.* **6(1),** 1–10.
4. Ballesteros, I., Ballesteros, M., Cabañas, A., Carrasco, J., Martin, C., Negro, M. J., Saez, F., and Saez, R. (1991), *Appl. Biochem. Biotech.* **28/29,** 307–315.
5. Chistyakova, T.A., Dedyukhina, E. G., and Eroshin, V. K. (1980), *Appl. Biochem. Microbiol.* **16,** 8.
6. Ingram, L. D. and Buttke, T. M. (1984), *Adv. Microl. Physiol.* **25,** 253–300.

7. Prashant, M. and Simminder, K. (1981), *Appl. Microbiol. Biotech.* **34,** 697–702.
8. Hayashida, S. and Ohta, K. (1978), *Agric. Biol. Chem.* **42,** 1139–1145.
9. Thomas, D. S., Hossack, J. A., and Rose, A. H. (1978), *Arch. Microbiol.* **117,** 239–245.
10. Janssens, J. H., Burris, N., Woodward, A., and Bailey, R. B. (1983), *Appl. Environ. Microbiol.* **45,** 598–602.
11. D'Amore, T. and Stewart, G. G. (1987), *Enzyme Microb. Technol.* **9,** 322–332.
12. Panchal, D. J. and Stewart, G. G. (1981), *Dev. Ind. Micribiol.* **22,** 711–715.
13. Casey, G. P., Magnus, D. A., and Ingledew, W. H. (1983), *Biotech. Lett.* **5,** 429–434.
14. Casey, G. P., Magnus, D. A., and Ingledew, W. M. (1984), *Appl. Environ. Microbiol.* **48,** 639–646.
15. D'Amore, T., Panchal, C. J., and Stewart, G. G. (1988), *Appl. Environ. Microbiol.* **54,** 110–117.
16. D'Amore, T., Panchal, C. J., Russell, I., and Stewart, G. G. (1988), *J. Ind. Microbiol.* **2,** 365–376.
17. Ballesteros, I., Oliva, J. M., Ballesteros, M., and Carrasco, J. (1993), *Appl. Biochem. Biotech.* **39/40,** 201–211.
18. Spindler, D. D., Wyman, C. E., and Grohmann, K. (1989), *Biotech.* **34,** 189–195.
19. Van Uden, N. (1984), *Adv. Microb. Physiol.* **25,** 195–251.
20. Dombek, K. M. and Ingram, L. O. (1986), *Appl. Environ. Microbiol.* **51,** 197–210.
21. Castanon, M. and Wilke, C. R. (1981), *Biotechnol. Bioeng.* **23,** 1365–1376.
22. Ooshima, H., Sakata, M., and Harano, Y. (1986), *Biotechnol. Bioeng.* **28,** 1727–1734.