Journal of Industrial Microbiology (1995) 15, 442–445
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# Effect of proteolipid on *Zymomonas* fermentation of 25% glucose media

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***Zymomonas mobilis*** **produces more than three times as many colony-forming units when grown in the presence of a combination of protein and lipid medium supplements than in unsupplemented cultures. The specific ethanol production rate is twice as fast, and the percent yield is higher (92% vs 82%), in supplemented than in unsupplemented broth. In addition, there is a change in the phospholipid composition of cells grown in the presence of supplements. Both materials are required for enhancement of fermentation and growth.**

**Keywords:** *Zymomonas mobilis*; ethanol; proteolipid; fermentation

## Introduction

In view of the potential for use of *Zymomonas mobilis* as an ethanol producer on an industrial scale [10,15], it is important to minimize its negative qualities, such as limited substrate range, if it is to be a serious competitor with yeast. In addition, positive qualities of *Z. mobilis*, such as rapid fermentation rate, should be maximized. Genetic approaches have begun to show promise in increasing substrate range [11,17]. One way to maximize the fermentation kinetics of *Zymomonas* is to improve the medium in which it is grown. It has long been known that *Saccharomyces* benefits from the presence of proteolipid (PL) additives in its fermentation medium [5,13]. The yeast shows improved ethanol production and tolerance, as well as increased biomass, when grown with these materials. Therefore, such additives were examined for their effects on *Z. mobilis*.

Membranes of cells are key targets of the toxic effects of ethanol [2,7]. Proteolipid supplementation of fermentation media causes changes in the lipids of *Saccharomyces* [6]. Therefore, the effects of PL on the membrane composition of *Zymomonas* were examined.

## Materials and methods

### Microorganism

*Zymomonas mobilis* strain CP4 was maintained on glucose-yeast extract agar [14]. Cultures were incubated in an atmosphere of $N_2$ at 28° C for 3 days, then stored at 4° C with biweekly subculturing.

### Medium and culture conditions

Glucose-yeast extract broth (GYEB) contained: yeast extract, 10 g $L^{-1}$; $KH_2PO_4$, 1 g $L^{-1}$; $(NH_4)_2SO_4$, 1 g $L^{-1}$; $MgSO_4$, 0.5 g $L^{-1}$; and glucose, 250 g $L^{-1}$ in the fermentation medium [14]. Glucose was sterilized separately from the other medium ingredients. When GYEB was supplemented with egg phosphatidylcholine (egg PC) and defatted ovalbumin [3], both were added to the salts/yeast extract portion of liquid medium before autoclaving it (except where filter sterilized albumin was added to cooled, sterile broth). PC was added, with stirring, to give a uniform emulsion, followed by albumin. PC and albumin concentrations were 5 g $L^{-1}$ and 10 g $L^{-1}$, respectively, unless otherwise indicated. The combination of PC + albumin will be referred to as proteolipid (PL). In some cases, other lipids or proteins were used, as noted in the results.

For fermentation studies, *Z. mobilis* was cultured in GYEB containing 10% glucose first, then inoculated into media containing 25% glucose. Cultures were grown in 125-ml Erlenmeyer flasks containing 100 ml of medium.

### Fermentation kinetics

Growth was followed by plate counts, using 10 mM Tris (pH 8) containing 50 mM $MgSO_4$ as diluent. A standard curve of plate count vs dry weight (linear in the range 25 $\mu$g $ml^{-1}$, $1.2 \times 10^7$ CFU $ml^{-1}$ to 1300 $\mu$g $ml^{-1}$, $4.9 \times 10^8$ CFU $ml^{-1}$ [$r = 0.997$]) was used to estimate biomass. Ethanol was determined by gas-liquid chromatography (GLC).

### Determination of lipids

Lipids were extracted by a small scale version of the method of Kates *et al* [9]. Phospholipids were separated by TLC on silica plates with chloroform : methanol : acetic acid (65 : 25 : 8, v : v : v). Phospholipids were quantified by a phosphate assay [1]. Fatty acids were esterified with 2% $H_2SO_4$ in methanol and examined by GLC.

### Determination of protein

Protein was assayed by the method of Lowry *et al* [12], with bovine serum albumin as the standard. When Triton and EDTA were present, samples were diluted and compared to standards containing these substances.

### Phospholipid to protein ratio

Phospholipid : protein was determined on isolated membranes [2], suspended in a minimum amount of 1% Triton X-100 in 3 mM EDTA (pH 7.3).

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Received 3 April 1995; accepted 19 July 1995

### *Cell wash procedure*

Cells were filtered through two layers of Whatman no 1 filter paper on a Buchner funnel to remove large particles of proteolipid. This was repeated with new filter paper. The filtrate was centrifuged at 4° C at 13 000 × *g* for 10 min. The pellet was washed 3× with 10 mM Tris buffer (pH 7) at 4° C.

To estimate the efficiency of the washing procedure, *Zymomonas* grown without proteolipid was mixed with defatted ovalbumin and soy PC, then treated as above. Since soy PC contains several fatty acids not found in *Zymomonas*, the effectiveness of removal of contaminating lipid could be determined by analyzing the lipids of these washed cells. Cells grown without proteolipid, then mixed with it to monitor the effectiveness of cell washing, had the same lipid composition as those never exposed to proteolipid.

### *Materials*

Yeast extract was from Oxoid (Columbia, MD, USA). Casein and agar were from Difco (Detroit, MI, USA). Wheat protein (gluten) and corn protein (zein) were from Nutritional Biochemical Company (Cleveland, OH, USA) and soya flour was from a local grocery store. Egg and soy PC were from Sigma Chemicals (St Louis, MO, USA) and ovalbumin was from Fisher Scientific (Springfield, NJ, USA). All other chemicals were reagent grade or better.

## Results

### *Effect of proteolipid on fermentation*

Proteolipid (ovalbumin + egg PC) added to the culture medium produced several stimulatory effects (Figure 1, Table 1). The maximum number of cells in supplemented GYEB was $1.6 \times 10^9$ CFU $ml^{-1}$, compared with $5.0 \times 10^8$ CFU $ml^{-1}$ in unsupplemented GYEB (Figure 1). The final ethanol concentration was 118 g $L^{-1}$ in supplemented GYEB, compared to 105 g $L^{-1}$ in the control. The specific

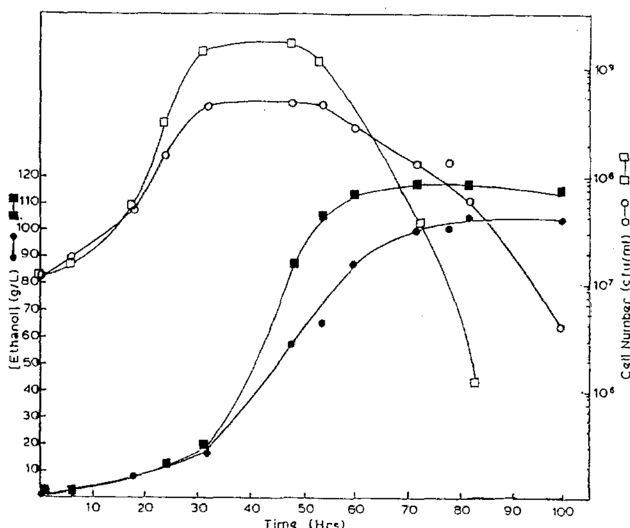


**Figure 1** Fermentation patterns of *Zymomonas mobilis* in GYEB containing 25% glucose with (□—□, ■—■) and without (○—○; ●—●) PL

**Table 1** Fermentation kinetics of CP4 in proteolipid supplemented and unsupplemented GYEB (25% glucose)

| Parameter | Unsupplemented | Supplemented[a] |
|---|---|---|
| P (g EtOH $L^{-1}$) | 104.90 ± 1.41 | 117.67 ± 6.45 |
| X (g cells $L^{-1}$)[b] | 1.26 ± 0.09 | 3.13 ± 0.61 |
| $Q_{p\ max}$ (g $g^{-1}$ $h^{-1}$) | 2.81 ± 0.73 | 6.91 ± 2.89 |
| $V_p$ (g $L^{-1}$ $h^{-1}$) | 2.47 ± 0.22 | 4.01 ± 0.39 |
| % of theoretical yield of ethanol | 82.27 ± 1.11 | 92.29 ± 5.06 |

[a]Broth was supplemented with 1% ovalbumin and 0.5% egg PC
[b]An approximation based on the maximum plate counts ($5.0 \times 10^8$ CFU $ml^{-1}$ for unsupplemented, and $1.6 \times 10^9$ CFU $ml^{-1}$ for supplemented medium)

ethanol production rate ($Q_{p\ max}$) was 2.5 times higher in medium containing proteolipid than in unsupplemented GYEB (Table 1).

The maximum growth rate and percent yield of product were higher in GYEB containing proteolipid than in unsupplemented broth. Thus, proteolipid stimulated fermentation and growth.

The rapid decline in viability of supplemented cells is not what might be expected if proteolipid renders *Zymomonas* cells ethanol tolerant. The death rate increases after a time (78 h, Figure 1) in supplemented broth. This occurs while the ethanol concentration is fairly stable.

Microscopic examination of samples revealed no increase in the number of clumped cells or cells attached to particles in diluted samples of the culture. Thus, the decline in plate counts is not due to artifacts introduced by aggregation of cells. Neither is it due to the dilution buffer, since the effect occurs with any diluent used (data not shown). Some diluents, such as 0.9% saline, yielded lower plate counts than described here, but the cells in supplemented medium still showed higher numbers of viable cells than the unsupplemented cells at high product concentration.

### *Effect of proteolipid composition*

In order to determine if proteolipid stimulation was restricted to only the combination of ovalbumin + egg PC, various other mixtures were examined as additives. Those chosen (casein + egg PC, casein + soy PC, zein + egg PC, gluten + egg PC, soybean meal, and defatted ovalbumin + soy PC) represent relatively cheap sources of proteolipid. All proteolipid mixtures tested produced effects similar to those already presented (data not shown).

### *Proteolipid compared to its parts*

A comparison of proteolipid to PC or protein alone showed that neither component by itself was sufficient to produce the same effect as whole proteolipid (Table 2). PC alone was inhibitory, while albumin alone had no effect on fermentation.

### *Effects of various proteolipid concentrations*

The ability of proteolipid to stimulate fermentation at lower concentrations was examined for two reasons. First, it would be more economical if less additive could be used to produce the same result. Second, separation of cells from

**Table 2** Comparison of proteolipid (PL) to defatted protein and to phospholipid in 25% GYEB

| Additive to GYEB | [EtOH] (g $L^{-1}$) after 48 h |
|---|---|
| None | 35.85 ± 1.25 |
| PL | 105.15 ± 1.85 |
| Defatted albumin[a] | 36.70 ± 2.29 |
| PC[b] | 21.07 ± 0.65 |

[a]1% ovalbumin
[b]0.5% egg phosphatidylcholine

**Table 3** Effect of proteolipid level on ethanol production

| Proteolipid concentration[a] (g $L^{-1}$) | Ethanol concentration[b] (g $L^{-1}$) |
|---|---|
| 0.00 | 35.85 ± 1.25 |
| 1.25 | 94.63 ± 6.21 |
| 2.50 | 90.35 ± 1.85 |
| 5.00 | 94.63 ± 7.19 |
| 7.50 | 91.55 ± 0.65 |
| 10.00 | 111.15 ± 1.85 |
| 15.00 | 105.15 ± 6.29 |

[a]2/3 defatted albumin + 1/3 egg PC, ie 15 g $L^{-1}$ = 10 g $L^{-1}$ albumin + 5 g $L^{-1}$ PC
[b]Ethanol concentrations after 48 h of fermentation

broth for biochemical analysis would be easier with less proteolipid. As Table 3 shows, concentrations of proteolipid as low as 1.25 g $L^{-1}$ gave a marked increase in ethanol production compared with unsupplemented GYEB. Similar results were obtained for soybean meal and untreated ovalbumin (data not shown).

### *Lipid composition of proteolipid-supplemented cells*

There was no measurable change in the major fatty acid content (Table 4) of *Z. mobilis* grown in the presence of proteolipid, whether soy or egg PC was used. Similarly, the phospholipid to protein ratio was 0.78 ± 0.11 $\mu$moles per mg without PL added to the medium and was 1.05 ± 0.36 $\mu$moles per mg when proteolipid was added. This difference was not significant. There was, however, a change in the phospholipid composition of cells grown in the presence of proteolipid. *Zymomonas* exhibited a decrease in phosphatidylethanolamine and an increase in PC and phosphatidylglycerol when grown with proteolipid (Table 5).

**Table 4** Fatty acids of CP4 grown in the presence and absence of PL[a]

| Fatty acid | With PL | Without PL |
|---|---|---|
| 14 : 0 | 7.50 ± 0.94 | 6.75 ± 2.00 |
| 16 : 0 | 18.44 ± 2.19 | 18.90 ± 2.40 |
| 18 : 1 | 72.19 ± 2.50 | 72.25 ± 3.00 |
| Others | 2.19 ± 0.63 | 2.25 ± 1.00 |

[a]PL = Proteolipid; base medium was GYEB

**Table 5** Major phospholipids of CP4 grown in GYEB in the presence and absence of proteolipid

| Phospholipid[a] | With PL | Without PL |
|---|---|---|
| PC | 42.50 ± 3.50 | 24.57 ± 1.58 |
| PE | 25.00 ± 1.75 | 64.70 ± 1.55 |
| PG | 25.75 ± 1.50 | 6.55 ± 1.02 |
| CL | 6.75 ± 0.75 | 4.18 ± 0.64 |

[a]PC = Phosphatidylcholine; PE = Phosphatidylethanolamine; PG = Phosphatidylglycerol; CL = Cardiolipin

## Discussion

Proteolipid has been added to *Saccharomyces* fermentation media to increase ethanol concentration and cell number [6]. The same effect was observed here with *Zymomonas*. The maximum number of colony forming units was 3.2 times higher in proteolipid-supplemented GYEB than plain GYEB. In addition, 12% more alcohol was produced in supplemented GYEB than in unsupplemented medium. Rates of growth and alcohol production were also increased in the supplemented broth.

There is one other report demonstrating proteolipid enhancement of *Zymomonas* fermentation [8]. In continuous culture, $Y_{p/s}$, biomass and effluent ethanol concentration increased when soy flour was used as the proteolipid source in the fermentation broth. In the present study, similar results were observed with batch cultures.

The ability to maintain viability in the presence of high ethanol concentrations is a characteristic that yeasts apparently gain when grown in the presence of proteolipid [6]. This is correlated with changes in the cellular lipid composition and slower leakage of UV-absorbing materials from supplemented cells [6].

*Zymomonas mobilis* does not appear to lose viability as quickly in supplemented broth as in unsupplemented GYEB. In unsupplemented GYEB, cell numbers began to decline when 90 g $L^{-1}$ ethanol accumulated in the broth. In contrast, cells grown in supplemented GYEB did not show a decrease in numbers until more than 100 g $L^{-1}$ ethanol had accumulated. This may reflect increased ethanol tolerance, and deserves further investigation.

We initially hoped to modify the fatty acid composition of *Zymomonas* membranes with lipid additives. This would allow the examination of the effects of various fatty acid profiles on responses by *Z. mobilis* to high ethanol concentrations. Unfortunately, supplementation with proteolipid did not yield such changes.

The increased metabolic rate demonstrated by the cells is probably due to the addition of a nutrient or nutrients when proteolipid is part of the culture medium. Perhaps proteolipid removes inhibitory substances, also.

The non-specificity of the additive requirements for stimulation indicates that, if cell feeding is the mode of action of proteolipid, it must be with simple subunits. Phospholipids can be incorporated into yeast cells when added as part of proteolipid [5], and this may be the case with *Zymomonas*.

In a medium supplemented with phospholipid and protein, *Zymomonas mobilis* increased the proportions of phos-

phatidylcholine and phosphatidylglycerol in cells at the expense of phosphatidylethanolamine (Table 5). Possibly, PC is incorporated into the cells, resulting in an elevated level of this lipid. *Zymomonas* does not normally exhibit an increase in PG in response to increasing ethanol concentrations in its environment [2]. This increase in PG may be a response to an increase of PC in the membranes, since bacteria apparently maintain tight regulation over the proportion of different phospholipid species in their membranes [4].

Future work should aim at elaborating the nature of the stimulation of fermentation by proteolipid. This should lead to improvements in *Zymomonas* fermentations through the directed use of more effective media.

## Definitions

$Q_{p\ max}$ = Maximum specific ethanol production rate (g product g cells$^{-1}$ h$^{-1}$)
$V_p$ = Volumetric productivity (g product L$^{-1}$ h$^{-1}$)
P = Concentration of product (ethanol) (g L$^{-1}$)
X = Biomass (g L$^{-1}$)

$Q_p$ was determined by the method of Stevnsborg and Lawford [16]. $V_p$ was calculated for the period of exponential growth and ethanol production.

## Acknowledgements

Publication D01901-1-95, NJ Agricultural Experiment Station (NJAES). Supported by Cooperative State Research Service grant 59-2341-1-2-099-0, by Hatch and NJAES funds.

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