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ETHANOL PRODUCTION BY RECOMBINANT *Escherichia coli* KO11 USING CRUDE YEAST AUTOLYSATE AS A NUTRIENT SUPPLEMENT

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SUMMARY

Crude yeast autolysate (10 g/l; 5.8 g solubles/l) supplemented with minerals and vitamins served as a nutrient supplement for ethanol production by the recombinant bacterium, *Escherichia coli* KO11. Ethanol production in this medium (46±1.7 g ethanol/l) was equivalent to that obtained using 5-15 g/l of purified, soluble, commercial protein hydrolysates (44-48 g ethanol/l). On site production of autolysates from spent yeast offers the potential for synergy between grain-based and lignocellulose-based ethanol plants.

INTRODUCTION

The production of fuel ethanol from lignocellulosic waste offers many potential economic and environmental benefits. Although methods are now available, the challenge of developing and commercializing a cost-effective process remains (Katzen and Fowler, 1994; von Sivers *et al.*, 1994). Genetically engineered bacteria such as *Escherichia coli* KO11 (Ingram *et al.*, 1991) and *Klebsiella oxytoca* P2 (Ingram *et al.*, 1995) have been shown to be effective biocatalysts for the fermentation of hemicellulose hydrolysates (Beall *et al.*, 1992) and mixed waste office paper (Brooks and Ingram, 1995), achieving ethanol levels of 40-50 g/l within 48 to 72 h. In most investigations, the refined commercial nutrients which have been employed are too costly for commercial ethanol production. Corn steep liquor is widely used as an inexpensive microbial nutrient (Atkinson and Mavituna, 1991) and supported excellent ethanol production by the pentose-fermenting yeast *Pichia stipitis* (Amartey and Jeffries, 1994). Recent studies have also demonstrated the utility of corn steep liquor (15 g dry weight/l) alone or in combination with crude yeast autolysate (4 g autolyzed yeast/l) for ethanol production by *E. coli* KO11 (Asghari *et al.*, 1996). However, corn steep liquor varies in quality and may contain toxins which require removal (Shah and Cheryan, 1995).

Autolysates of spent yeast have been shown to be an effective nutrient for very high gravity wheat fermentations (Jones and Ingledew, 1994a). Yeast from grain-based ethanol plants could also serve as an inexpensive source of nutrients for companion plants which produce ethanol from lignocellulose. Previous studies have described a procedure for the fractionation of yeast into food products (Kollar *et al.*, 1992). Using a minor modification of this procedure, a crude yeast autolysate was prepared and used to develop a nutrient medium for ethanol production by *E. coli* KO11.

MATERIALS & METHODS

Chemicals: Pressed yeast cakes were purchased from a local bakery. Inorganic salts, except molybdic acid (technical), were reagent grade. Salts, Difco Tryptone, Difco Soytone and Difco Yeast Extract were purchased from the Fisher Scientific Company (Norcross, GA). Spezyme Fan^{tm} (protease) was generously donated by Genencor International (South San Francisco, CA). Chloramphenicol was purchased from the Sigma Chemical Company (St. Louis, MO). **Organism and culture conditions:** *E. coli* KO11 was used in these studies and contains chromosomally integrated *Zymomonas mobilis* genes (*pdc, adhB*) for ethanol production (Ohta *et al.*, 1991). Unless noted otherwise, media for pH-stats contained per liter: macronutrient mineral salts (2 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, and 2 g NaCl); 0.5 g $MgSO_4 \cdot 7\ H_2O$, 11 mg $FeCL_3 \cdot 6\ H_2O$; vitamins (25 μg cyanocobalamin, 100 μg calcium pantothenate, 50 μg pyridoxine·HCl, and 500 μg thiamine·HCl), complex nutrient (5 g Difco product or 10 g crude yeast autolysate), 100 g glucose, and 40 mg chloramphenicol. A chloramphenicol stock (1000X) was prepared in 70% ethanol. Inocula for pH-stats were grown for 18 h at 30°C in LBG medium (Luria and Delbruck 1943) (per liter: 50 g glucose, 5 g Difco Yeast Extract, 10 g Difco Tryptone, 5 g sodium chloride) and 40 mg chloramphenicol. Cells were harvested by centrifugation (5,000 X g, 5 min) for use as inocula (158 mg dry weight/l). **Preparation of yeast autolysate:** A crude yeast autolysate was prepared in batches by a modification of the method described by Kollar *et al.* (1992). Sufficient water was added to 200 g of yeast cake (70% moisture; 200 g dry weight/l), 3 g NaCl, and 16 g ethanol (20 ml) to produce a total volume of 300 ml. A glass marble was added to aid agitation (60 cycles/min) and the mixture incubated in a sealed 500-ml flask for 24 h at 50°C. This served as a 20-fold concentrate and was stored frozen at -20°C. Autolysate was Pasteurized (15 min, 90°C) immediately prior to use (final concentration of 10 g autolyzed yeast/l). **Fermentation in pH-stats:** Modified Corning Fleakers™ (500 ml total volume, 350 ml working volume) were used as vessels for pH-stats (Beall *et al.*, 1991). These were immersed in a 35°C water bath and agitated with a magnetic stir bar (100 rpm). Broth pH was controlled during fermentation by the automatic addition of 2N KOH and was not allowed to fall below pH 6.0. Ethanol, base consumption, and pH were measured at 24 h intervals. **Analyses:** Ethanol was measured by gas liquid chromatography using isopropanol as an internal standard (Ohta *et al.*, 1991). Ethanol yields were calculated after correcting for dilution by added base and for ethanol present at the time of inoculation (yeast autolysate and chloramphenicol stock). Yields are expressed as a percentage of the maximum theoretical yield (51 g ethanol per 100 g glucose) and were not corrected for unmetabolized sugar. Moisture content was measured after drying for 48 h at 70°C. Free amino nitrogen (FAN) was measured using glycine as a standard (European Brewery Convention, 1987).

RESULTS & DISCUSSION

Yeast autolysate Kollar *et al.* (1992) developed an optimal batch procedure for yeast autolysis (100 g autolyzed yeast/l) based on the solubilization of protein. This procedure included freshly prepared autolysate (prior batch), 1% NaCl and 5% (w/v) ethanol. Initial experiments, using ethanol production by *E. coli* KO11 (48 h) as a bioassay, indicated that the yeast concentration could be doubled to produce a nutrient which was equivalent on a dry weight basis. Using this higher yeast concentration eliminated any benefit from the addition of autolysate form a prior batch. Approximately 58% of the yeast dry weight was solubilized during the preparation of 20% yeast autolysate.

Media optimization A series of experiments was conducted with 20%-yeast autolysate to determine the optimize the supplements (macronutrient salts, magnesium, trace metals, and vitamins) for ethanol production. An initial mixture of 7 trace metals was completely replaced by $FeCl_3$ (11 mg/l). Similarly, a mixture of 10 vitamins was reduced to 4 vitamins (per liter: 25 μg cyanocobalamin, 100 μg calcium pantothenate, 50 μg pyridoxine·HCl, and 500 μg thiamine·HCl). Figure 1A and Table 1 illustrates the small but significantly detrimental effect of omitting either of these supplements.

Inorganic sources of nitrogen (2 g $(NH_4)_2SO_4$/l) and phosphorus (1 g K_2HPO_4/l) were also beneficial (Figure 1; Table 1). A small further increase in ethanol production was obtained with a 50% increase in the level of ammonia, phosphate, or both. Reducing either macronutrient or magnesium resulted in lower ethanol concentrations and lower yields. As a complex nutrient, 20%-yeast autolysate was clearly superior to 10%-yeast autolysate (Table 1). The more concentrated nutrient also has the obvious advantage of reducing the volumes which must be processed. Omission of yeast autolysate reduced fermentation rates by over 50% (Figure 1A & 1B). Reductions in the level of yeast autolysate (25% and 50%) also reduced the rates of ethanol production and ethanol yields.

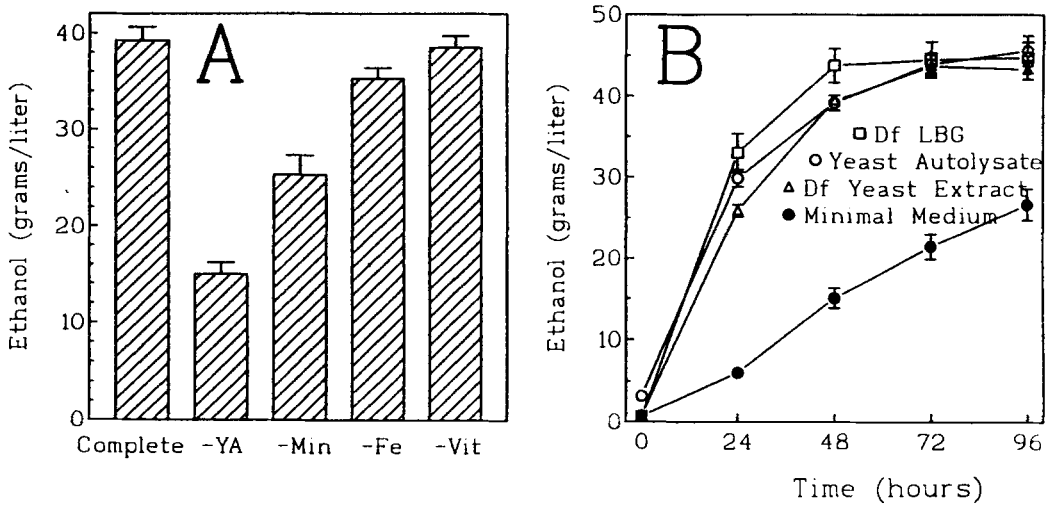

Figure 1. Ethanol production in pH-stats using yeast autolysate media. Results are presented as averages with standard deviations from three or more experiments. A. Effect of supplements on ethanol production (10% glucose, 48 h). Complete medium is described in the **MATERIALS and METHODS** and contains 50 ml yeast autolysate (YA)/l, macronutrient salts (Min), $FeCl_3 \cdot 6H_2O$ (Fe), and 4 vitamins (Vit). Individual components were omitted where indicated. B. Comparison of yeast autolysate medium to Difco (Df) nutrients. Results with minimal medium plus vitamins (no complex supplements) are also included.

Table 1. Effects of nutrients on fermentation (100 g glucose/l)

Nutrients[a,b,c]	n[d]	Ethanol (g/l)	Base[e] (ml/l)	Yield (% theoretical)
1. LBG medium (Difco)	23	44.7±1.9	62.9	92
Difco nutrient (g/l) + salts + Mg + Fe + vitamins				
2. Yeast Extract (5)	4	43.7±0.9	62.9	90
3. Tryptone (5)	3	47.9±0.5	51.4	97
4. Soytone (5)	6	44.8±1.7	54.3	91
10%-Yeast Autolysate (g yeast/l) + salts + Mg + Fe + vitamins				
5. yeast autolysate (10)	3	44.3±0.5	77.1	83
20%-Yeast Autolysate (g yeast/l) + salts + Mg + Fe + vitamins				
6. yeast autolysate (10)	21	46.0±1.7	68.6	90
7. #6 minus Fe	3	44.7±1.2	62.9	87
8. #6 minus salts	3	29.7±5.4	97.1	58
9. #6 minus vitamins	6	41.3±3.2	91.4	83
10. #6 minus yeast autolysate	4	26.7±1.9	71.4	50
11. #6 plus protease[f]	3	44.2±1.5	85.7	88
12. #6 with 1.5X Mg	2	43.5	71.4	86
13. #6 with 1.5X NH_4	2	47.6	54.3	93
14. #6 with 1.5X PO_4	2	48.7	57.1	95
15. #6 with 1.5X [NH_4+PO_4]	4	47.0±1.1	62.9	92
16. #6 with 0.5X Mg	2	42.5	94.3	85
17. #6 with 0.5X NH_4	2	42.7	114.3	87
18. #6 with 0.5X PO_4	2	44.5	80.0	88
19. #6 with 0.75X yeast autolysate	4	42.9±4.6	62.9	85
20. #6 with 0.5X yeast autolysate	5	35.8±2.3	77.1	72
21. #20 with protease[d]	3	39.9±0.5	94.3	82
22. #20 with 1.5X NH_4	2	41.4	65.7	83
23. #20 with 1.5X [NH_4+PO_4]	2	38.9	62.9	77

[a] Abbreviations: Mg, $MgSO_4 \cdot 7H_2O$; Fe, $FeCl_3 \cdot 6H_2O$; NH_4, $(NH_4)_2SO_4$; and PO_4, K_2HPO_4.

[b] Average initial ethanol concentrations in fermentation broth were as follows: 0.7 g/l for LBG and Difco products; 5.3 g/l for 10%-Yeast Autolysate; 3.0 g/l for 20%-Yeast Autolysate (2.4 g/l for 0.75X and 1.9 g/l for 0.5X).

[c] Omitted salts were replaced by an equivalent weight of NaCl.

[d] Number of fermentation trials.

[e] 2 N NaOH added to maintain pH during fermentation.

[f] Yeast autolysate prepared with Spezyme FAN protease (4 ml/l) as a supplement.

The addition of Spezyme protease (4 ml/l) during autolysis did not appear to improve the nutritional value of autolysate when assayed at a 1:20 dilution (Table 1). When tested at 1/2-strength, however, autolysate containing protease supported higher rates of ethanol production and a higher product yield than control autolysate. The addition of 50% higher levels of ammonia and phosphate also improved fermentations with half-strength hydrolysate but yields remained below that obtained with full strength autolysate.

Ethanol yield with crude yeast autolysate and optimal supplements was equivalent to that obtained with LBG (Figure 2B; Table 1) albeit with a slightly slower rate of ethanol production. The nutritional value of crude yeast autolysate was compared to Difco products (Table 1). When compared using the same supplements, 20%-yeast autolysate diluted to 10 g autolyzed yeast/l was equivalent to 5 g/l Yeast Extract or Soytone but less effective than Tryptone. The FAN contents of these fermentation media were examined as a possible basis for the nutritional differences (Jones and Ingledew, 1994b; Thomas and Ingledew, 1990). LBG (475 mg/l) contained the highest level of FAN, followed by protease-supplemented yeast autolysate (225 mg/l), Yeast Extract (177 mg/l), yeast autolysate (168 mg/l), Tryptone (149 mg/l) and Soytone (83 mg/l). Although it is clear that complex nutrients containing amino acids stimulate fermentation, factors other than FAN content must also contribute to the effectiveness.

It is difficult to produce high ethanol concentrations from most lignocellulosic biomass with current technology. Final ethanol concentrations of 40-50 g/l have been achieved and are realistic (Katzen and Fowler, 1994). From 16-20 liters of broth must be fermented and processed to produce a single liter of ethanol, emphasizing the importance of an inexpensive nutrient media. As illustrated above, crude yeast autolysate, minerals, and a small amount of vitamins can serve as an excellent nutrient for ethanologenic *E. coli* KO11. This media may also be useful for other biotechnology applications.

On site production of autolysate from spent yeasts offers an opportunity for synergy between grain-based and biomass-based ethanol production. Spent yeasts from grain-based ethanol plants are typically blended with other residues to produce animal feed. Spent yeasts could be "borrowed" for use as a bacterial nutrient. Evaporation of the stillage from bacterial fermentations should recover yeast amino nitrogen together with additional amino nitrogen from bacterial biosynthesis.

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