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# Soy-based medium for ethanol production by *Escherichia coli* KO11

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**An optimized soy-based medium was developed for ethanol production by *Escherichia coli* KO11. The medium consists of mineral salts, vitamins, crude enzymatic hydrolysate of soy and fermentable sugar. Ethanol produced after 24 h was used as an endpoint in bioassays to optimize hydrolysate preparation. Although longer fermentation times were required with soy medium than with LB medium, similar final ethanol concentrations were achieved (44–45 g ethanol $L^{-1}$ from 100 g glucose $L^{-1}$). The cost of materials for soy medium (excluding sugar) was estimated to be \$0.003 $L^{-1}$ broth, \$0.06 $L^{-1}$ ethanol.**

**Keywords:** soy; hydrolysate; nutrient; fermentation; ethanol; amino acids

## Introduction

The development of cost-effective methods for the conversion of lignocellulose to ethanol offers many potential opportunities to solve environmental waste problems and to replace petroleum-based automotive fuels [7,15]. Ethanologenic *Escherichia coli* [8,9] have been developed as biocatalysts for the fermentation of hemicellulose syrups [2]. Final ethanol concentrations are limited to 40–50 g $L^{-1}$ due to product inhibition, requiring the processing of up to 20 L of broth per liter of ethanol. As with yeast [11,16], the rate of fermentation and ethanol yield are higher when complex nutrients are added [12]. Amino nitrogen has been identified as being of particular importance [6,10,16]. Organic nitrogen, amino acids, and commercial proteases are marketed as supplements for the yeast-based ethanol industries [11].

Corn steep liquor (15 g $L^{-1}$; \$0.20 $kg^{-1}$ dry weight) can serve as an excellent nutrient supplement for ethanol production by pentose-fermenting yeast [1] and *E. coli* KO11 [2]. However, the quality of corn steep liquor is variable and sufficient quantities may not be available in all areas. Commercial, purified protein hydrolysates are too costly (\$3.50–\$18.00 $kg^{-1}$ in bulk) for ethanol production. Soy and other proteins are widely used as nutrients for many industrial processes [3,17]. This study describes the preparation of a soy-based medium for ethanol production by *E. coli* KO11.

## Materials and methods

*E. coli* KO11 is a recombinant derivative of *E. coli* B in which the *Zymomonas mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) have been integrated into the host chromosome [14]. Tryptone™, Soytone™ and Yeast Extract™ were obtained from Difco (Detroit, MI, USA). Soybeans, soy flour, and soy meal were purchased locally (approximately 8% moisture). The particular size of soybeans and soy meal was reduced with a commercial coffee grinder prior to digestion. Spezyme Fan™ protease was generously provided by Genencor International (South San Francisco, CA, USA).

Soy hydrolysates prepared by different methods were tested in shaken, 250-ml flasks (50 ml broth, 35°C). Media for flask-fermentations (and for initial studies in pH-stats) contained per liter: 50 ml of test hydrolysate, 100 g glucose, 2 g $(NH_4)_2SO_4$, 1 g $K_2HPO_4$, 2 g NaCl, 0.25 g $MgSO_4 \cdot 7H_20$, 5.4 mg $FeCl_3 \cdot 6H_2O$, 0.4 mg $ZnCl_2 \cdot 4H_2O$, 0.4 mg $CoCl_2 \cdot 6H_2O$, 0.4 mg molybdic acid (tech), 0.2 mg $CuCl_3 \cdot 2H_2O$, 0.2 mg $CaCl_2 \cdot 2H_2O$, 0.1 mg $H_3BO_3$, 2 mg choline chloride, 2 mg nicotinic acid, 1 mg thiamine · HCl, 0.1 μg cyanocobalamin, 0.2 μg *p*-aminobenzoic acid, 0.2 μg biotin, 0.4 μg calcium pantothenate, 0.2 μg folic acid, 0.2 μg pyridoxine · HCl, and 0.2 μg riboflavin. Flasks were inoculated by transferring a small colony from solid media. Ethanol produced after 24 h was used as the endpoint. Ethanol was determined by gas chromatography [4]. Free amino nitrogen was determined spectrophotometrically using glycine as a standard [5].

Combinations of mineral and vitamin supplements were tested in pH-stats (350 ml working volume, pH 6.0, 35°C, 100 rpm) [4]. These were inoculated from broth cultures to an initial density of 165 mg cell dry weight $L^{-1}$. Optimized soy-based medium contained per liter: 50 ml crude soy hydrolysate (9.2 g soy solids $L^{-1}$) or 5 g Tryptone™, 100 g glucose, 2 g $(NH_4)_2SO_4$, 1 g $K_2HPO_4$, 2 g NaCl, 0.25 g $MgSO_4 \cdot 7H_20$, 10.8 mg $FeCl_3 \cdot 6H_2O$, 0.25 mg thiamine · HCl, 0.1 μg calcium pantothenate, 0.05 μg pyridoxine · HCl, and 0.02 μg cyanocobalamin.

## Results and discussion

### Soy hydrolysate

Ethanol production by *E. coli* KO11 (flask cultures) was used as a bioassay to optimize the denaturation and

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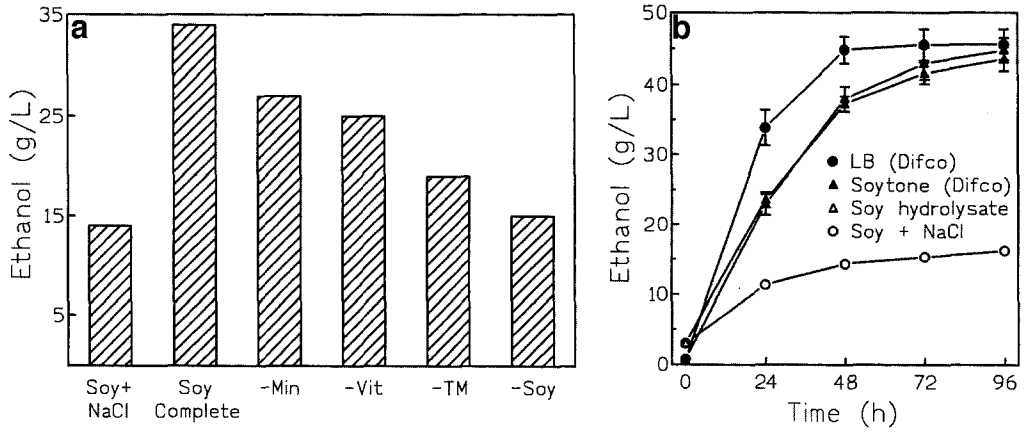


**Figure 1** Ethanol production in soy hydrolysate-based media. (a) Effect of nutrient supplements on ethanol production after 48 h. Soy + NaCl contained only soy hydrolysate and 5 g sodium chloride $L^{-1}$. Soy complete contained 50 ml soy flour hydrolysate $L^{-1}$ (Soy), macronutrient salts (Min), vitamins (Vit), and trace metals (TM). Components were omitted as indicated. (b) Comparison of optimal soy hydrolysate-based medium to LB and Soytone (5 g $L^{-1}$)-based medium. Standard deviations ($n \geq 6$) are indicated by error bars for LB, Soytone, and Soy hydrolysate media.

hydrolysis of soy with Spezyme FAN™ (data not shown). The resulting procedure for the production of a 20-fold nutrient concentrate (300 ml) is: 1) combine 60 g soy flour, ground soybeans or soy meal (approximately 8% moisture) with 220 ml tap water in a 500-ml screw-capped Erlenmeyer flask (approximately pH 8); 2) denature by heating to 95°C for 2 h with reciprocal shaking; 3) cool to room temperature and adjust to pH 9 with 10 N sodium hydroxide; 4) add 20 ml of 95% ethanol and 1.2 ml of Spezyme Fan™; 5) mix thoroughly and incubate for 18 h at 50°C with reciprocal shaking. Hydrolysates were stored frozen until needed and pasteurized for 15 min at 90°C immediately before use. Inclusion of ethanol in the hydrolysate appeared to increase the efficacy of pasteurization. Approximately 50% of soy dry weight was solubilized with a free amino nitrogen content of 1.0 g $L^{-1}$.

### *Soy-based fermentation medium*

Ethanol production by *E. koli* KO11 (pH-stats) was used to optimize vitamin and mineral supplements for soy hydrolysate-based medium (9.2 g $L^{-1}$ total soy, 4.6 g $L^{-1}$ soy solubles). Figure 1a shows the beneficial effects of supplements on ethanol production. Subsequent experiments indicated that $FeCl_3$ could fully replace the mixture of trace metals. Only 4 of the 10 vitamins tested were beneficial. When compared on the basis of solubles, soy hydrolysate prepared as described was equivalent to commercial Soytone™ as a nutrient for ethanol production (44–45 g ethanol $L^{-1}$). Neither fermentation rate nor ethanol yield was increased significantly by increasing the levels of vitamins or minerals above that in the optimized soy medium.

Fermentations were also conducted in modified LB medium [13] (per liter: 10 g Tryptone™, 5 g Yeast Extract™, 5 g NaCl, and 100 g glucose) for comparison. No combination of vitamins and minerals was found which allowed fermentations with soy hydrolysate (4.6 g soy solubles $L^{-1}$) to reach completion as rapidly as fermentations with LB medium (15 g soluble hydrolysate $L^{-1}$). The completion of fermentations in soy medium may be slower due to the increased demand for amino acid biosynthesis. Final ethanol concentrations achieved with both media were nearly equivalent (Figure 1b).

This study demonstrates that crude soy hydrolysates can be used as an effective nutrient supplement for bacterial ethanol fermentations. Market values for soybeans and Spezyme Fan™ are approximately \$200 metric $ton^{-1}$ and \$3 $L^{-1}$, respectively. Using current values for minerals and vitamins, the materials (excluding sugar) for optimized soy fermentation broth are estimated to cost \$0.003 $L^{-1}$ (\$0.06 $L^{-1}$ of ethanol), equal to that of corn steep liquor-based medium [2].

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## References

1 Amartey S and TW Jeffries. 1994. Comparison of corn steep liquor with other nutrients in the fermentation of D-xylose by *Pichia stipitis* CBS 6054. Biotechnol Lett 16: 211–214.
2 Asghari A, RJ Bothast, JB Doran and LO Ingram. 1996. Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli* strain KO11. J Ind Microbiol 16: 42–47.
3 Atkinson B and F Mavituna. 1991. Biochemical Engineering and Biotechnology Handbook, 2nd edn. Stockton Press, New York, NY.
4 Beall DS, K Ohta and LO Ingram. 1991. Parametric studies of ethanol production from xylose and other sugars by recombinant *Escherichia coli*. Biotechnol Bioeng 38: 296–303.
5 European Brewery Convention. 1987. Free amino nitrogen. In: Analytica-EBC (4th edn). pp E141–E142, European Brewery Convention, Zurich, Switzerland.
6 Guimaraes WV, GL Dudey and LO Ingram. 1992. Fermentation of sweet whey by ethanologenic *Escherichia coli*. Biotechnol Bioengin 40: 41–45.
7 Hohmann N and CM Rendleman. 1993. Emerging technologies in ethanol production. US Department of Agriculture Information Bulletin Number 663, pp 1–17.
8 Ingram LO, DS Beall, GFH Burchardt, WV Guimaraes, K Ohta, BE Wood and KT Shanmugam. 1995. Ethanol production by recombinant hosts. US Patent No. 5 424 202.
9 Ingram LO, T Conway, DP Clark, GW Sewell and JF Preston. 1987. Genetic engineering of ethanol production in *E. coli*. Appl Environ Microbiol 53: 2420–2425.

10 Jones AM and WM Ingledew. 1994. Fermentation of very high gravity wheat mash prepared using fresh yeast autolysate. Biores Technol 50: 97–101.
11 Jones AM and WM Ingledew. 1994. Fuel alcohol production: appraisal of nitrogenous yeast foods for very high gravity wheat mash fermentation. Process Biochem 29: 483–488.
12 Lawford HG and JD Rousseau. 1991. Ethanol production by recombinant *Escherichia coli* carrying genes from *Zymomonas mobilis*. Appl Biochem Biotechnol 28/29: 221–236.
13 Luria SE and M Delbruck. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28: 491–511.
14 Ohta K, DS Beall, JP Mejia, KT Shanmugam and LO Ingram. 1991. Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl Environ Microbiol 57: 893–900.
15 Sheehan JJ. 1994. Bioconversion for production of renewable transporation fuels in the United States: a strategic perspective. In: Enzymatic Conversion of Biomass for Fuels Production, ACS Symposium Series 566 (Himmel ME, JO Baker and RP Overend, eds), pp 1–53, American Chemical Society, Washington, DC.
16 Thomas KC and WM Ingledew. 1990. Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mashes. Appl Environ Microbiol 56: 2046–2050.
17 Zabriskie DW, WB Armiger, DH Phillips and PA Albano. 1988. Traders' Guide to Fermentation Media Formulation. 1988. Traders Protein, Memphis, TN.