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Fermentation of Orange Peel Hydrolysates by Ethanologenic *Escherichia coli*

Effects of Nutritional Supplements†

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ABSTRACT

Orange peel, an abundant byproduct of the citrus processing industry, is converted to a mixture of glucose, galacturonic acid, fructose, arabinose, galactose, and xylose by hydrolysis with mixed pectinase and cellulase enzymes. All these sugars can be fermented to ethanol or ethanol and acetic acid by the recombinant bacterium *Escherichia coli* KO11. The fermentation efficiency is improved by the addition of yeast extract, tryptone, mixed amino acids, corn steep liquor, or, by proteolytic digestion of endogenous proteins. Batch fermentations of supplemented peel hydrolysate containing 111 g/L of initial total sugars produced 35–38 g/L of ethanol in 48–72 h and a 75–85% yield.

Index Entries: Recombinant *E. coli* B; ethanol production; citrus byproducts.

INTRODUCTION

Orange peel and membranes are abundant byproducts of the citrus processing industry *(1,2)*. Although these byproducts are currently dried in the US and sold as cattle feed *(3)*, they do not command a very high price and could be used for the production of fuel ethanol. More than one million dry tons of these byproducts accumulate in two main processing countries, the US and Brazil *(4)*, with additional supplies being potentially available in other countries where citrus production and processing are undergoing expansion.

Previous studies from our laboratory *(5,6)* reported that *Escherichia coli* KO11 *(7–11)* efficiently ferments all neutral sugars and galacturonic acid in orange peel hydrolysates to ethanol and smaller amounts of acetic and lactic acids in the pH range of 5.8–6.5 and at 34–37°C, when these hydrolysates are supplemented with

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1.25 g/L of yeast extract and 2.5 g/L tryptone. Supplementation with these complex nutrients increases the cost of fermentation. Therefore, we have undertaken a more detailed study of nutritional requirements of this fermentation with the aim to develop more practical methods for improvement of the nutritional content of these hydrolysates.

MATERIALS AND METHODS

Bacterial Strains and Media

A recombinant strain of *E. coli* B (ATCC11303), strain KO11 was a gift from Lonnie O'Neal Ingram, University of Florida, Gainesville, FL. Cultures were maintained as frozen stock (–75°C) in 40% glycerol.

Culture Medium

The medium for fermentation experiments consisted of filtered Valencia orange peel hydrolysate prepared by hydrolysis of orange processing byproducts using a mixture of commercial cellulase, pectinase, and β–glucosidase enzymes as described previously *(5,6,12,13)*. The filtrate was partially neutralized by addition of calcium carbonate to pH 5.5, and the precipitated solids were removed by filtration through a 1.2-μm glass fiber filter. The medium and nutritional supplements were filter-sterilized (0.45-μm membrane filter), and chloramphenicol was added to a final concentration of 60 mg/L. The supplementation of the media is described below in the text (*see* Table 1). Filter-sterilized proteinase preparations were added to some fermentations at concentrations recommended in a previous publication *(11)* or at concentrations described later in this article. The proteinase preparations were added immediately after inoculation.

Fermentation Experiments

Fermentations were carried out in pH-controlled, stirred fermenters at pH 6.0, as described previously *(6)* at a temperature of 35°C. Fermentations were inoculated to an initial cell density of 0.11 g dry cell wt/L.

Analyses

Growth was monitored by filtration of culture aliquots through 0.45-μm membrane filters and drying the filters to constant weight at 70°C.

Concentrations of ethanol, acetic, lactic, and galacturonic acids were measured by ion-moderated partition chromatography and refractive index detection as described previously *(5,12,13)*. Neutral sugars were determined by a separate ion-moderated partition chromatography using a Pb^{2+} column *(5,12,13)*. Values for ethanol and acid yields were corrected for changes in volume caused by addition of base (2*N* KOH) and smaller amounts of acid (2*N* HCl) used for pH control and for the amount of ethanol introduced with the inoculum.

Materials and Reagents

Streptomyces griseus proteinase (Type XIV, P5147, approximate specific activity 4 U/mg solid) and *Bacillus polymyxa* proteinase (Type XV, P5647, approximate SA 0.4 U/mg solid) were purchased from Sigma (St. Louis, MO). Vitamin assay

Table 1
Fermentation Summary

Supplement	Concentration, g/L	Fermentation time: 24 h Ethanol[a] concentration, g/L	24 h Ethanol[b] yield, % theor.	24 h Volumetric productivity, g/L/h	48 h Ethanol concentration, g/L	48 h Ethanol yield, % theor.	48 h Volumetric productivity, g/L/h	72 h Ethanol concentration, g/L	72 h Ethanol yield, % theor.	72 h Volumetric productivity, g/L/h	Final[c] cell mass, g/L
None		20.9	43.4	1.00	31.7	68.5	0.72	35.0	72.1	0.54	4.2
Tryptone + yeast extract	2.5 + 1.25	25.2	54.9	1.05	36.4	78.5	0.79	38.1	75.1	0.53	6.2
Tryptone	2.5	27.4	59.9	1.13	36.9	84.7	0.80	36.7	84.9	0.53	4.2
Yeast extract	1.25	25.6	55.7	1.05	35.3	79.8	0.75	35.7	81.3	0.51	4.2
Ammonia	10	20.4	39.4	0.70	24.8	60.8	0.54	—	Not done	—	6.6
Phosphate	10	13.0	24.7	0.49	20.9	43.6	0.42	33.5	72.8	0.46	6.4
Phosphate and ammonia	5 + 5	24.2	50.0	0.89	28.4	70.1	0.63	—	Not done	—	6.3
Casamino acids (vitamin free)	1	23.4	50.5	0.93	35.0	78.3	0.72	35.5	81.6	0.50	4.1
Amino acid mixtures:											
#1[d]	0.1 (ea.)	23.3	46.1	0.92	33.3	68.8	0.69	—	Not done	—	4.1
#2[e]	0.1	19.4	44.6	0.75	34.1	69.7	0.69	—	Not done	—	3.9
#3[f]	0.1	24.7	50.3	1.00	34.2	71.2	0.71	—	Not done	—	3.9
#4[g]	0.1	19.6	48.4	0.75	32.5	66.2	0.67	—	Not done	—	3.9

[a]Not corrected for dilution. [b]Calculated from the initial monosaccharide content. [c]Dry basis. [d]Cys, Met. [e]His, Leu, Ileu,Val, Lys. [f]Phe, Tyr, Trp, Thr, Pro. [g]Glu, Ser, Ala, Asp, Arg.

casamino acids were manufactured by Difco (Detroit, MI). Corn steep liquor was a kind gift of Bioenergy International (Gainesville, FL). It assayed at 56% of dry total solids.

RESULTS AND DISCUSSION

The fermentation of orange peel hydrolysates was investigated at 35°C and pH 6.0, since previous studies *(6)* indicated that these conditions are optimal for this fermentation. Filtered peel hydrolysates diluted to 80% strength before the fermentation had the following final concentrations in wt% (standard deviations) of individual monomeric sugars: glucose 4.22 (0.39), fructose 2.39 (0.23), arabinose 0.89 (0.16), galactose 0.58 (0.04), and galacturonic acid 3.02 (0.25), respectively. Average concentration of total neutral sugars was 8.08 and of total sugars 11.1 wt%, respectively. Dilution of peel hydrolysates by addition of inoculum, nutritional supplements, and water decreased the initial concentration of sugars to a level allowing efficient fermentation of all neutral sugars and formation of relatively concentrated ethanol solutions *(6)*. Formation of additional monomeric sugars during fermentation was not observed. Average results of ethanol production at 24, 48, and 72 h in different media and final dry cell concentration are compiled in Table 1. A comparison of results in the first two rows of Table 1 indicates that unsupplemented peel hydrolysate exhibits only marginal levels of nutritional deficiency, because only the rate of ethanol formation is slower when compared to fermentation of supplemented peel hydrolysate. Supplementation with yeast extract or tryptone alone indicates that either supplement improves fermentability of peel hydrolysate to the same extent as richer media utilized previously *(6)*. Addition of phosphate and ammonia either alone or in combination did not have a stimulatory effect on this fermentation, while an addition of vitamin-free casamino acids appeared to be as effective as supplementation with richer media. We also investigated supplementation with pools of amino acids designed to test for auxotrophic requirements *(14)*. The results in Table 1 do not indicate significant stimulation by any particular mixture of amino acids or auxotrophic requirement for any particular amino acid by *E. coli* KO11.

Since supplementation with mixed amino acids provided the most effective improvement in fermentation performance, we investigated supplementation with different concentrations of casamino acids. The results (not shown) indicate that approx 1 g/L of casamino acids provides adequate stimulation of fermentation of peel hydrolysates by *E. coli* KO11.

Supplementation with a corn steep liquor can provide a cheap source of amino acids and other nutrients *(15)*. The results obtained at different levels of supplementation with corn steep liquor are summarized in Fig. 1. Optimal level of supplementation appears to be at 1.5% (v/v) of corn steep liquor or 0.86% of corn steep liquor solids, because at higher concentrations, the corn steep liquor seemed to inhibit the fermentation.

Another avenue for supplementation of orange peel hydrolysates with amino acids can be provided by proteolysis of endogenous proteins *(11)*. We evaluated two protease preparations used successfully for stimulation of cheese whey fermentation by *E. coli* KO11 *(11)*. The results summarized in Fig. 2 indicate that proteinase from *S. griseus* provided efficient supplementation at 20–100 U/L, whereas proteinase from *B. polymyxa* appears to be somewhat inhibitory to these fermentations.

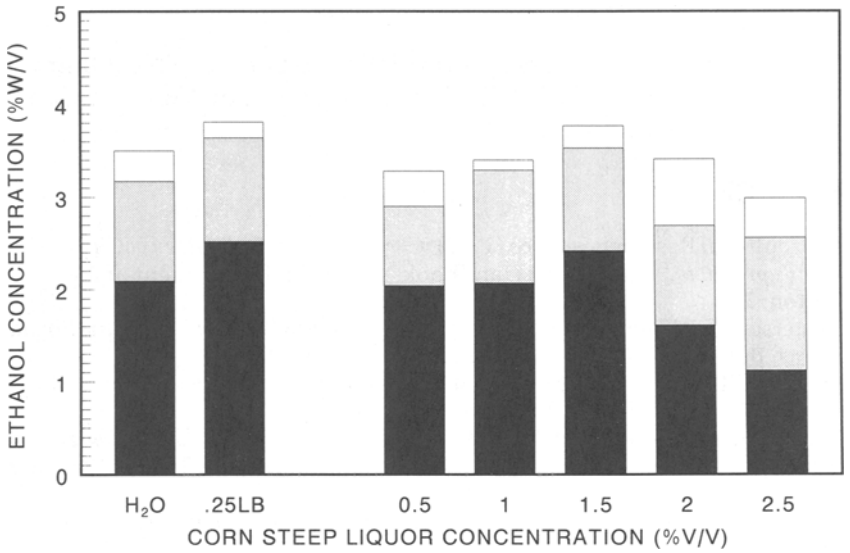

Fig. 1. Effects of supplementation of orange peel hydrolysate with corn steep liquor on ethanol production by *E. coli* KO11. ■, 24 h; ▒, 48 h; ▨, 72 h; H_2O, no supplement; 0.25LB, 2.5 g/L tryptone and 1.25 g/L yeast extract.

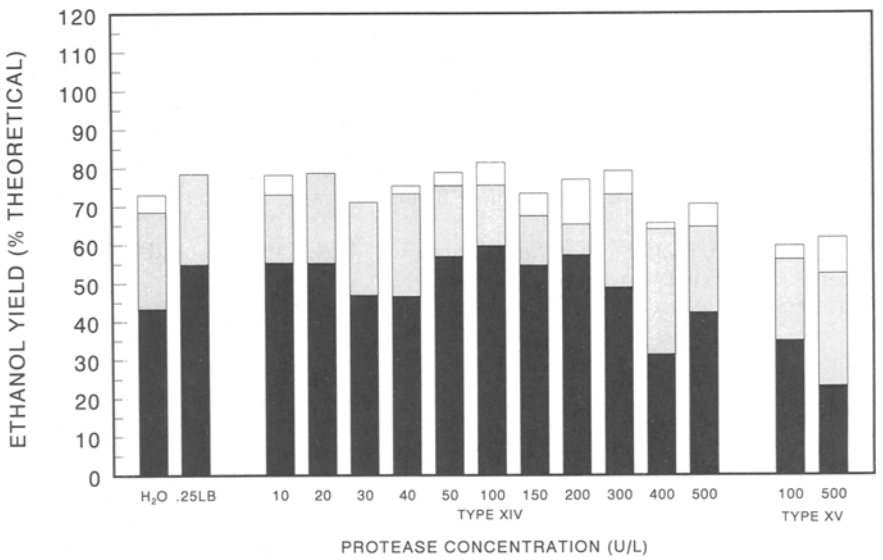

Fig. 2. Effects of supplementation of orange peel hydrolysate with proteases from *S. griseus* (Type XIV) and *B. polymyxa* (Type XV). The bars are coded as in Fig. 1.

CONCLUSIONS

The fermentation of orange peel hydrolysates by *E. coli* KO11 is stimulated by supplementation with low amounts of amino acids and peptides. The stimulation can be readily accomplished by supplementation with mixtures of amino acids, peptides, corn steep liquor, or by proteolysis of endogenous proteins in orange peel hydrolysates.

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