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Production of ethanol from sugars in wood hydrolysate by *Fusarium oxysporum*

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Wood hydrolysate used for ethanol production by two strains of *Fusarium oxysporum* contained 2.3% (w/v) reducing sugars (xylose and glucose). Ethanol production at the optimum reducing sugar concentration of 54.8 g/l medium, at pH 5.5, and 30°C was 12.3 g/l and 11.7 g/l by *F.oxysporum* D-140 and NCIM-1072, respectively in shake flasks during 96 h fermentation. The maximum production of ethanol under optimum cultural conditions, and in the presence of yeast extract plus minerals, was 13.2 g/l medium by *F.oxysporum* D-140 over 108 h fermentation.

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In pulp and paper industries, cellulose and part of the lignin are used for the production of finished products whereas hemicellulose, an easily hydrolysable component (varies in different tree woods from 3 to 25%), is a major waste product (Jeffries 1983). The predominant hemicellulosic sugar of agricultural residues is xylose. Flickinger (1980) suggested that pentoses from hemicelluloses could be an economical source of carbohydrates for conversion to liquid fuel. Hemicellulosic sugars in acid hydrolysates of hardwoods and agricultural residues could become important feedstock for the production of ethanol and other chemicals by microbial process.

Certain fungi e.g., *Fusarium lini* and *F.oxysporum* can ferment xylose to ethanol (Batter & Wilke 1977; Vikari *et al.* 1981). Xylose fermentation was studied by Suihko & Enari (1981) in order to provide the basis for an economically feasible ethanol production using sugars derived from biomass. Although xylose fermentation by filamentous fungi is generally regarded as a slow process, one of the best organisms available for xylose fermentation is *Fusarium oxysporum* (Suihko 1984).

In the present investigation, the fermentation of sugars in wood hydrolysate by a filamentous fungus *Fusarium oxysporum* has been investigated for the production of ethanol.

Materials and Methods

Substrate and Treatment

Wood hydrolysate was obtained from Century Pulp and Paper Factory, Lalkuan, India. It was concentrated to 20% of its volume by evaporation in a water bath at 60°C for 24 h. The liquor was then neutralized with $Ca(OH)_2$ to pH 5.5 and filtered. This solution was used for all experiments.

Cultures

Fusarium oxysporum NCIM-1072 was from the National Collection of Industrial Microorganisms, National Chemical Laboratories, Pune, India and *F.oxysporum* D-140 was from the laboratory of Dr M.L. Suihko, Finland.

L'hydrolysat de bois utilisé pour la production d'éthanol par deux souches de *Fusarium oxysporum* contenait 2.3% (poids/vol.) de sucres réducteurs (xylose et glucose). La production d'éthanol, à la concentration optimum en sucres réducteurs de 54.8 g par litre de milieu à pH 5.5 et à 30°C était de 12.3 g/l et 11.7 g/l respectivement chez *F. oxysporum* D-140 et NCIM-1072, en flacons agités pendant 96 h de fermentation. La production maximum d'éthanol, dans les conditions optimum de culture, et en présence d'extrait de levure et de minéraux était de 13.2 g par litre de milieu chez *F. oxysporum* D-140 en 108 h de fermentation.

Adaptation of Cultures

The basal growth medium contained (g/l): glucose (20), $NaNO_3$ (3.5), yeast extract (1) and KH_2PO_4 (2). It was supplemented with neutralized wood hydrolysate (pH 5.5) at 10, 20 and 50% (v/v) concentrations and sterilized at 121°C for 15 min. Test cultures were inoculated in the medium containing 10% (v/v) wood hydrolysate and incubated at 30°C for 48 h and were subsequently subcultured serially in media containing 20 and 50% (v/v) wood hydrolysate and allowed to grow for 48 h at 30°C in each. The cultures finally adapted to 50% wood hydrolysate were used to develop inoculum for fermentation.

Preparation of Inocula

Inocula were grown in medium containing (g/l): glucose (40), yeast extract (1), $NaNO_3$ (3.4), KH_2PO_4 (2), $CaCl_2.2H_2O$ (0.4) and $MgSO_4.7H_2O$ (0.3). The *F.oxysporum* strains were inoculated and incubated at 30°C for 3 days.

Effect of Reducing Sugar Concentrations in Wood Hydrolysate and pH

The original wood hydrolysate was concentrated to a fifth. Each 100 ml flask received 20 ml of different reducing sugar concentrations ranging from the original to 5 fold concentrated liquor and sterilized at 121°C for 15 min. Flasks also received 1 ml each of separately sterilized 2.5% (w/v) yeast extract and a stock mineral solution containing (g/l): $NaNO_3$ (7.5), KH_2PO_4 (50), $CaCl_2.2H_2O$ (10) and $MgSO_4.7H_2O$ (7.5). The pH was adjusted to 4.5, 5.0, 5.5 or 6.0. Each flask was inoculated with 3 ml of *F.oxysporum* D-140 or NCIM-1072 and incubated at 30°C on rotary shaker (160 rev/min) for 5 days. Samples were analysed for ethanol production and residual reducing sugars.

Ethanol Production at Optimum Conditions

Concentrated wood hydrolysate, 200 ml, was autoclaved in a 1 l flask. To this was added 10 ml of separately sterilized solution of yeast extract (2.5%) and minerals (see above) and then inoculated with 30 ml of *F.oxysporum* D-140. The flasks were incubated as before, sampled periodically and analysed for ethanol production, mycelial dry weight and residual reducing sugars.

Analytical Determinations

Samples of fermented slurry were centrifuged at 2100 *g* for 25 min. The supernatant was decanted, adjusted to pH 7 and used for ethanol and residual sugar assayed. The cell debris was washed twice with warm distilled water (45 ± 2°C) re-centrifuged and the mycelial biomass dried at 60°C to constant weight.

Ethanol was estimated colorimetrically following the method of Reid & Salmon (1955) and extrapolated against the absolute ethanol standard curve prepared according to the method of Ranganna (1977).

Reducing sugars were determined by dinitrosalicylic acid (DNS) method of Miller (1959).

Chromatographic Analysis of Sugars

Wood hydrolysate was concentrated in a flash evaporator, decolorized with activated charcoal and a sample separated by paper chromatography using n-butanol/acetic acid/propan-2-ol/water (20:3:3:9, by vol). Sugars were revealed by spraying with diphenylamine reagent (Bailey & Burns 1960). For quantitative analysis of sugars, 100 mg wood hydrolysate was chromatogrammed and visualized as before. The position of each spot was marked on unsprayed paper, cut out, shredded, suspended in 30 ml distilled water in a 100 Erlenmeyer flask and agitated for 12 h on rotary shaker. The mixture was then held at 60°C for 30 min, filtered through Whatman No. 1 paper and washed twice with distilled water (at 55 ± 2°C) to ensure maximum recovery of sugars. Each sugar

extract was then flash evaporated to 5 ml and the content of xylose and glucose estimated by the dinitrosalicylate method of Miller (1959).

Results and Discussion

The pH of the wood hydrolysate was 3.5 and the reducing sugar content was 2.3% (w/v). Xylose and glucose were present, respectively, at 18.5 and 4.5 g/l wood hydrolysate. Other sugars, arabinose, galactose, and mannose, were not detected.

Table 1 shows the effect of reducing sugar concentration on the fermentation of wood hydrolysate to ethanol by *F.oxysporum* D-140 and NCIM-1072 during 120 h fermentation. The maximum conversion of reducing sugars in wood hydrolysate to ethanol was 28.1% and 27.4% (g/100 g consumed reducing sugars) with *F.oxysporum* D-140 and NCIM-1072 strains, respectively, at an initial concentration of 54.8 g reducing sugars/l medium. Though ethanol obtained at reducing sugar concentrations of 88.6 g/l and 73.2 g/l was higher than at 54.8 g/l medium, the ethanol yield was lower. Consequently, a reducing sugar concentration (in wood hydrolysate) of 54.8 g/l medium was chosen for maximum ethanol yield and used in all further experiments.

The effect of different pH values on fermentation of reducing sugars in wood hydrolysate (at 54.8 g/l medium) to ethanol showed a direct correlation between increase in pH from 4.5 to 5.5 and sugar utilization (Table 2). Maximum values were at pH 5.5 and were 12.6 ethanol medium and 11.9 ethanol medium using *F.oxysporum* D-140 and NCIM-1072, respectively. Maximum ethanol yields were thus 28.8% and 27.6% (w/w), respectively. Our results agree with those of Suihko (1984) who reported pH 5.5 was the optimum for xylose fermentation by *F.oxysporum*.

Figure 1 shows the production of ethanol during the growth of *F. oxysporum*

Table 1. Effect of reducing sugar concentrations in wood hydrolysate on ethanol produced by *Fusarium oxysporum* D-140 and NCIM-1072 at pH 5.5 and temperature 30°C in shake flasks.

Initial reducing sugar concentration (g/l medium)	Time (h)	*F. oxysporum* D-140			*F. oxysporum* NCIM-1072		
		Reducing sugar utilized (g/l)	Ethanol produced (g/l)	Ethanol yield (g/100g reducing sugar utilized)	Reducing sugar utilized (g/l)	Ethanol produced (g/l)	Ethanol yield (g/100g reducing sugar utilized)
18.4	48	5.5	1.2	22.2	5.3	1.2	22.3
	96	14.7	3.7	25.1	14.6	3.6	24.8
	120	15.3	3.7	23.9	14.8	3.5	24.0
36.6	48	11.6	2.6	22.4	11.4	2.6	22.9
	96	29.2	7.7	26.5	28.9	7.6	26.2
	120	29.8	7.1	23.8	29.7	7.1	23.9
54.8	48	17.4	4.1	23.3	16.5	3.9	23.6
	96	43.7	12.3	28.1	42.9	11.7	27.4
	120	44.7	12.3	27.3	43.7	11.7	26.7
73.2	48	21.9	4.8	22.6	20.8	4.5	21.9
	96	58.1	14.4	24.7	57.9	14.0	24.1
	120	59.4	14.0	23.1	58.2	13.3	22.8
89.6	48	23.5	4.7	20.1	23.3	4.6	19.8
	96	63.6	14.8	23.2	63.1	14.5	23.0
	120	64.2	14.4	22.5	63.7	13.9	21.8

Table 2. Effect of pH on ethanol production from sugars in wood hydrolysate by *Fusarium oxysporum* D-140 and NCIM-1072 at 30°C in shake flask (at a reducing sugar concentration of 54.8 g/l medium).

pH	Time (h)	*F. oxysporum* D-140		*F. oxysporum* NCIM-1072	
		Reducing sugar utilized (g/l)	Ethanol produced (g/l)	Reducing sugar utilized (g/l)	Ethanol produced (g/l)
4.5	48	11.0	1.9	9.9	1.6
	96	28.9	5.3	27.6	5.0
5.0	48	14.2	2.8	13.9	3.5
	96	36.4	7.6	35.0	7.1
5.5	48	17.5	4.1	16.3	3.9
	96	43.7	12.6	42.9	11.9
6.0	48	15.0	3.2	15.6	3.1
	96	37.9	8.8	38.5	8.5

D-140.The maximum concentration of ethanol, 13.2 g/l medium with ethanol yield of 28.8% (w/w), was reached after 108 h. There was no evidence for diauxic growth (on glucose and xylose), thus agreeing with the previous results of Suihko (1984).

After fermentation (120 h) about 20% of the original sugars still remained in the medium. Since prolonged fermentation resulted in loss of ethanol, we did not attempt further utilization of the substrate.

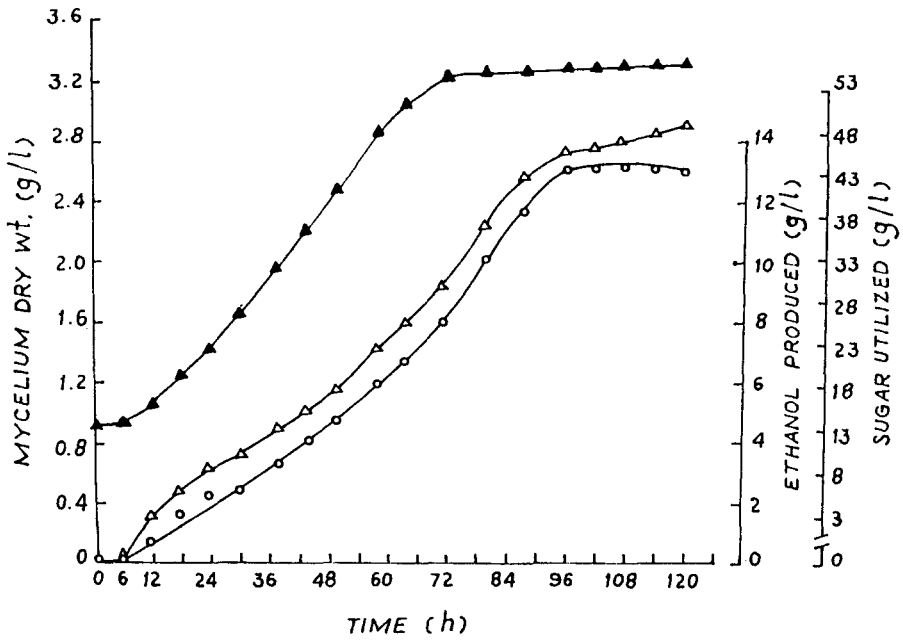

Figure 1. Effect of incubation time on ethanol production (O), sugar utilization (△), and mycelium yield (▲) from wood hydrolysate by *Fusarium oxysporum* D-140 during 120 h fermentation.

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