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D-XYLOSE CATABOLIZING ENZYMES IN NEUROSPORA CRASSA AND THEIR RELATIONSHIP TO D-XYLOSE FERMENTATION

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Summary

The induction of xylose reductase (XR) and xylitol dehydrogenase (XD) activities by D-xylose under different fermentation conditions was investigated in ***Neurospora crassa***. The induction of NADPH-linked XR preceded NADH-linked XR and the ratio of NADH to NADPH-linked XR activity displayed variation from 0.02 to 0.2 suggesting the presence of two separate enzymes. Aerobic conditions were required by ***N. crassa*** for cell growth but not for ethanol production. Maximum ethanol of 0.3 g/g of D-xylose was produced when shifted to semiaerobic condition, where high NADH-linked XR and NAD-linked XD activities were observed.

Introduction

The utilization of cellulose as well as hemicellulose is important for the economic production of liquid fuels from biomass (Biely, 1985). D-xylose, the predominant sugar of hemicellulose is not so readily utilized as D-glucose for the production of ethanol by microorganisms. The bacteria, yeast and moulds differ in their mode of conversion of D-xylose to D-xylulose, the initial step in the xylose metabolism (Jeffries, 1983). Few yeasts such as ***Candida*** sp. (Jeffries, 1981), ***Pachysolen tannophilus*** (Dekker, 1982) and ***Pichia stipitis*** (Dellweg, *et al.*, 1984) are known to utilize D-xylose for the production of ethanol. It has been reported that the key to observed differences among yeasts with respect to anaerobic xylose fermentation may be due to the coenzyme specificity of the initial reaction.

Numerous fungi are known to degrade both cellulose and hemicellulose but only a few exhibit both depolymerase and fermentatitve activities (Jeffries, 1983). We have earlier shown the production of extracellular cellulase and xylanase activities and direct conversion of biomass to ethanol by ***N. crassa*** (Rao *et al.*, 1983; Mishra *et al.*, 1984; Deshpande *et al.*, 1986). The biotechnological implications of ethanol production by ***N. crassa*** prompted us to investigate the enzymes involved in xylose ferementation. The understanding

of the enzyme systems will help to increase the overall efficiency of xylose fermentation. The present paper reports the presence of xylose reductase (XR, EC 1.1.1.21) and xylitol dehydrogenase (XD, EC 1.1.1.14) in *N. crassa* NCIM 870. The relationship between the enzyme activities and xylose fermentation is also discussed.

Materials and Methods

Microorganism and culture medium: *N. crassa* NCIM 870 was obtained from National Collection of Industrial Microorganisms, Pune, India. *N. crassa* was grown in Vogel's minimal medium (Vogel *et al.*, 1984) containing 2% xylose as the carbon source at 28°C.

Fermentation conditions The organism was grown aerobically by inoculating a heavy spore suspension in a 500 ml flask containing 50 ml of medium and 2% xylose as the carbon sourse. The flask were shaken at 220 rpm (24 h) and were transferred to aerobic, non-aerated (semiaerobic) and anaerobic conditions. Under the aerobic conditions the organism was grown in 1 L flask at 220 rpm. The experiments under non-aerated condition were carried out by transferring the contents of the growth flask to 100 ml stoppered flask with a capillary opening at the top containing 2% D-xylose. For anaerobic conditions the contents of the growth flask were transferred asceptically to 100 ml flask with 2% xylose. The flasks were gassed with nitrogen before and after the transfer of the cell mass. Ethanol was determined by gas chromatography as described earlier (Rao *et al.*, 1983).

Preparation of cell extract: The cell mass was suspended in 50 mM sodium phosphate buffer (pH 7.2) containing 2 mM each of B-mercaptoethanol, phenyl-methyl-sulphonyl-fluoride and magnesium chloride. The cells were sonicated for 10 min. and the cell debris was removed by centrifugation (15,000 x g, at 4°C for 20 min).

Enzyme assays and protein determination: Enzymes were assayed spectrophotometrically at 30°C. The reaction rates were linearly proportional to the amount of extract added and were corrected for endogenous NAD(P)H consumption or production.

Xylose reductase : The assay mixture (1 ml) contained 100 mM sodium phosphate buffer, pH 6.3, 0.05 mM NADPH or NADH and an appropriate amount of cell extract. The reaction was started by the addition of D-xylose to a final concentration of 200 mM and A_{340} decrease was measured.

Xylitol dehydrogenase: The assay mixture (1 ml) contained 50 mM Tris-HCl buffer, pH 8.2, 10 mM $MgCl_2$, 0.2 mM NAD^+ and an appropriate amount of cell extract. The reaction was started by the addition of xylitol to a final concentration of 200 mM and the A_{340} increase was measured.

Enzyme units are defined as nmoles of nicotinamide nucleotide reduced or oxidized min^{-1}. Specific activities are expressed as units mg^{-1} of protein. Protein was measured as described by Lowry *et al.*, (1951).

Results and Discussion

XR and XD activities were induced by D-xylose in *N. crassa*. D-glucose was an effective repressor and low enzyme activities were obtained (Table 1). The similar extent of repression (90%) of XR and XD by D-glucose suggests that these enzymes may be under co-ordinate control. In case of *Pichia stipitis* a similar type of glucose repression was observed whereas in *Pachysolen tannophilus* the XD activity was repressed to greater extent than XR activity (Bicho *et al.*, 1988). During the growth under aerobic conditions the specific activity of NADPH-linked XR increased more rapidly and at an earlier stage compared to the NADH-linked enzyme (Fig. 1). The specific activity of NAD-linked XD was considerably higher compared to the NADP-linked activity. The traces of NADP-linked XD activity may be due to the affinity of XR for the cofactor (reverse reaction) (Bruinenberg *et al.*, 1984) When aerobically grown cultures of *N. crassa* were shifted to semiaerobic stage, NADPH-linked XR remained constant whereas there was an increase in NADH-linked XR and NAD-linked XD activities (Fig. 2). The levels of the enzymes under anaerobic conditions (data not shown) were similar to those determined under semiaerobic condition.

Table 1: Induction of NADPH/NADH-linked XR and NAD dependent XD activities in N. crassa.

Carbon source	Enzyme activities (Units.mg^{-1})		
	XR		XD
	NADPH-linked	NADH- linked	NAD-linked
Xylose (2%)	585	120	620
Xylose (1%) + Glucose (1%)	99	18	99
Glucose (2%)	54	10	62

N. crassa was grown under aerobic condition for 24 h and shifted to semiaerobic condition (48 h). Enzyme activities were determined as described in Materials and Methods.

Aerobic batch cultures of *N. crassa* did not produce ethanol from D-xylose. However, when shifted to semiaerobic condition resulted in the formation of ethanol. It was observed that the ratio of NADH- to NADPH-linked XR

activities varied with the period under semiaerobic condition. Maximum ethanol was obtained when high NADH-linked XR and NAD-linked xylitol dehydrogenase activities were observed. The presence of NADH-linked XR probably prevents the imbalance of the NAD^+/NADH redox system, thus allowing the conversion of xylose to ethanol. The corelation between the fermentative ability of yeast and its NADH-linked XR activity has been shown by Bruinenberg*et al.*,(1984).

The induction of NADPH-linked XR preceeded NADH-linked XR. The ratio of NADH-to NADPH-linked XR activities displayed variation from 0.02 to 0.2 suggesting the presence of two different enzymes (Fig. 1). This is also supported by the differential stability of the enzymes. NADPH-linked XR was more stable and retained 100% and 70% activity at 4°C (24 h) and 40°C (2 min.) whereas NADH-linked XR lost 80 and 100% of its activity at 4 and 40°C respectively. Ditzelmuller *et al.*, (1985) have isolated two xylose reductases showing specificity to NADPH and NADH respectively. However, the dual coenzyme specificity of XR in *P. tannophilus* has been reported by Verduyn *et al.*, (1985).

Identification of fungal systems which would ferment cellulose and hemicellulose directly to ethanol is significant commercially but have never been seriously considered for developing process technology. This is understandable in view of the more rapid fermentation of sugars to ethanol by yeast. Generally, yeast are unable to metabolize cellulose and hemicellulose. The ability of *N. crassa*, a single organism to produce ethanol from biomass is attractive from economical point of view. In the present studies we have demonstrated the presence of NADPH- and NADH-linked XR and NAD-linked XD in *N. crassa* NCIM 870 and the crucial role of NADH-linked XR for alcoholic fermentation of xylose.

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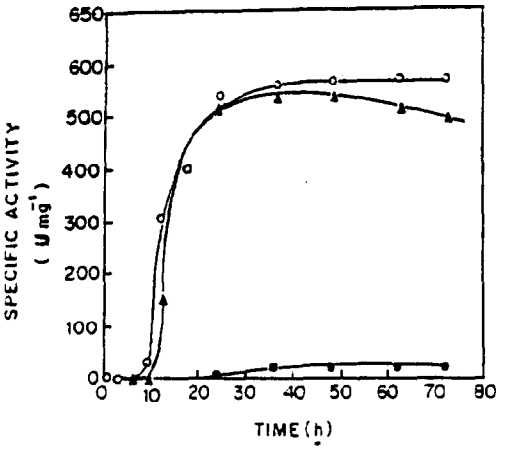

FIG. 1

Fig. 1:

The induction of xylose catabolizing enzyme by N. crassa under aerobic conditions.

NADH- (●), NADPH- (o) linked XR and NAD-linked XD (▲) activities.

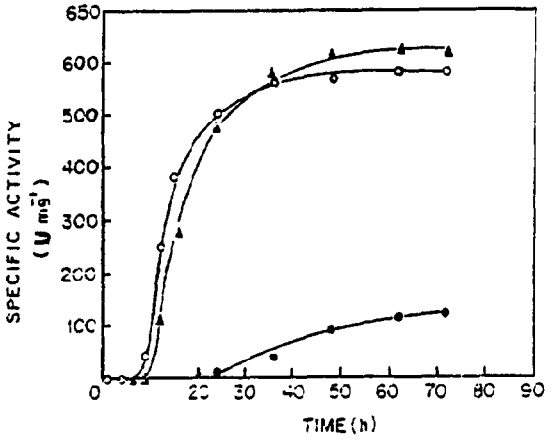

FIG. 2

Fig. 2:

The levels of enzyme activity under semiaerobic conditions.

NADH- (●), NADPH- (o) linked XR and NAD-linked XD (▲) activities.

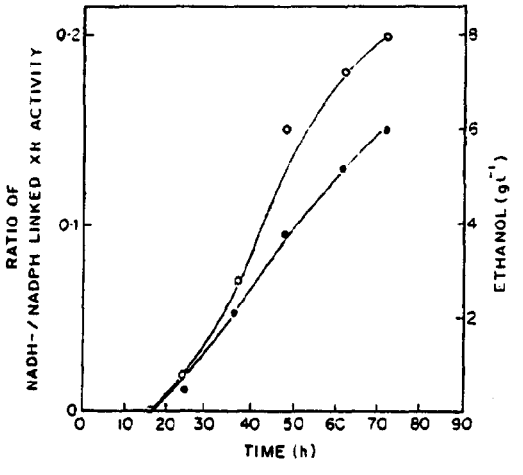

FIG. 3

Fig. 3:

Correlation between ratio of NADH-to NADPH-linked XR activity and ethanol production by N. crassa under semiaerobic conditions.

Ratio of NADH-/NADPH-linked XR activity (o) and ethanol (●).