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XYLITOL FORMATION BY *Candida boidinii* IN OXYGEN LIMITED CHEMOSTAT CULTURE

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SUMMARY

Reduction of xylitol by *Candida boidinii* NRRL Y-17213 occurs under conditions of an oxygen limitation. The extent to which substrate is converted to xylitol and its coproducts (ethanol, other polyols, acetic acid). and the relative flow of substrate to energetic and biosynthetic pathways is controlled by the degree of oxygen limitation.
With decrease in oxygen consentration in the inlet gas, for a constant dilution rate of 0.05 1/h. the specific oxygen uptake rate decreased from 1.30 to 0.36 mmol/gh Xylitol was not produced at specific oxygen uptake rates above 0.91 mmol/gh. Upon shift to lower oxygen rates, specific xylitol production rate increased more rapidly than specific ethanol production rate:

INTRODUCTION

Xylitol, a naturally occurring five-carbon polyol is currently used as a sugar substitute. On the large scale it is still produced chemically, although a microbial production is recently becoming very attractive. Among the microorganisms, the best xylitol producers are xylose fermenting yeasts, particularly ones belonging to the genus *Candida* (Barbosa *et al.*, 1988, Vongsuvanlert and Tani, 1989, Meyrial *et al.*, 1991, Horitsu *et al.*, 1992, Nolleau *et al.*, 1993, Silva and Afschar, 1994).
Xylose metabolism by yeasts has been studied in batch and continuous cultures. However, due to their accuracy, data collected from continuous culture, are more useful than batch data for obtaining the quantitative information needed for construction of a metabolize model (Alexander *et al.* 1988). Under oxygen limited conditions, according Pirt's model, the specific growth rate μ, which equals to the dilution rate in the continuous culture, is limited by the oxygen uptake rate: $\mu = Y_{cm\cdot O2} (q_{O2} - m_{O2})$.

Xylose fermenting yeasts do not grow under anoxic conditions and do not ferment when fully aerobic. Fermentation and growth occur simultaneously only under oxygen limitation (Alexander *et al.* 1989). Aeration is necessary because it supplies the culture with oxygen, which is required for yeast growth even under fermentation for the synthesis of unsaturated fatty acids and ergosterol (Kuriyama and Kobayashi, 1993).

The xylitol producing yeasts *Candida boidinii* NRRL Y-17213, which has been the subject of our investigations (Vandeska *et al.* 1995a) exhibits the same characteristics as the other xylose fermenting yeasts. Therefore, to determine the effect of oxygen on xylose metabolism by this yeast, we carried out continuous cultivation under oxygen limitation and excess of xylose.

MATERIALS AND METHODS

Microorganism

Candida boidinii NRRL Y-17213, was maintained on agar slants at 4°C. The slant medium, (YPG), contained (g/l): yeast extract, 10; bactopeptone, 20; glucose, 20 and agar 20.

Inoculum preparation

The medium for inoculum preparation was as described before (Vandeska *et al.* 1995a). Xylose solution (120 g/l) was sterilized separately by autoclaving and added aseptically to the medium. The inoculum was prepared by transferring a loopful of cells from 3-day-old YPG slant into 25 ml of medium in a 125 ml baffled Erlenmeyer flask plugged with foam and cultivated with shaking at 300 rev/min for 24 h at 30°C. 5 ml of this culture was transferred into 50 ml of medium in a 250 ml baffled Erlenmeyer flask and cultivated under the same conditions for 48 h. This culture was used as an inoculum in concentration of 2.5 % (v/v) of the working volume in the fermentor.

Culture medium

The composition of the fermentation medium is given in Table L All four solutions were sterilized separately. The vitamins were filter sterilized, while the others were autoclaved and afterwards mixed aseptically.

Cultivation

Continuous cultivation was carried out using a 2-1 fermenter (Biostat, Braun). The working volume was controlled at 1.5 l. Agitation was performed by four 6-bladed flat turbine impellers and was varying from 350 to 950 rev/min. The medium feeding rate was controlled to give a dilution rate of 0.05 l/h using peristaltic pump (Ismatic instruments). The pH was automatically maintained at 5.5 with 2 M KOH or H_2SO_4 solutions. The temperature was controlled at 30°C. Using mass flow controllers (Aalborg AFC 2600) the inlet gas flow rate was changing between 20 ml/min and 2 l/min. Oxygen concentration in the inlet gas was changed from 20.9 % (air) to 51.9 % in a mixture of oxygen and nitrogen. The composition of the exit gasses were monitored by an oxymeter and infra-red carbon dioxide analyzer (Mine Safety Appliances Company). Dissolved oxygen tension, DOT, was measured with a polarographic oxygen electrode (Ingold). Steady state conditions were considered to be established after 5 turnovers of the medium.

Analytical methods

Samples were taken every 4 to 6 hours and centrifuged. After washing the residues twice, with distilled water, they were dried for cell mass determination at 102°C. Xylose, xylitol, and other polyols were determined by high-performance liquid chromatography while ethanol and acetic acid were analized by gas chromatography as described elsewhere (Sreenath *et al.,* 1986).

Oxygen uptake rate (OUR) measurement was performed by monitoring the outlet O_2 concentration and subtracting it from the O_2 concentration observed in the inlet gas.

Table 1. The composition of the fermentation medium

Components	*Amount per litre*	*Components*	*Amount per litre*
Solution 1		***Solution 3***	
$MgCl_2 \cdot 6H_2O$	500 mg	Ca-pantothenate	20 mg
citric acid	500 mg	thiamine.HCl	5 mg
$CaCl_2 \cdot 2H_2O$	50 mg	piridoxine.HCl	20 mg
$FeCl_3$	25 mg	nicotinic acid	5 mg
$MnCl_2 \cdot 4H_2O$	31 mg	p-aminobenzoic acid	1 mg
$ZnCl_2$	6 mg	biotin	0.1 mg
$CuCl_2 \cdot 2H_2O$	0.75 mg	***Solution 4***	
$CoCl_2 \cdot 6H_2O$	2 mg	xylose	100 g
$Na_2MoO_4 \cdot 2H_2O$	1.3 mg	inositol	0.1 g
KI	0.35 mg	antifoam	1.0 g
Boric acid	2 mg		
Solution 2			
K_2SO_4	178 mg		
KH_2PO_4	4.9 g		
urea	6.5 g		
casamino acids	2.5 g		

RESULTS AND DISCUSSION

Yeast differ in their partitioning of metabolism between respiration and fermentation that is, between their use of oxygen and organic compound as terminal electron acceptors (Alexander *et al.*, 1990). The flow rates of the substrate to each of these pathways are determined by the availability of oxygen.

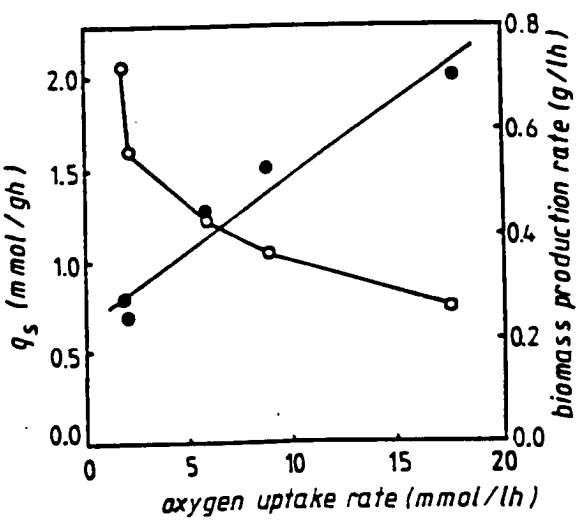

Figure 1

Effect of oxygen uptake rate on biomass production rate (●) and specific xylose uptake rate (○) in oxygen. limited chemostat culture of *C. boidinii;* dilution rate. 0.05 1/h.

Fig. 1 shows biomass production rate and specific xylose consumption rate by *C. boidinii* versus oxygen uptake rate for a constant dilution rate of 0.05 1/h. Over the investigated

oxygen uptake rates, a linear relation seems to exist between biomass growth rate and oxygen uptake rate. The slope of the line gives the bruto yield of biomass on oxygen, Y_{cmO2}, and equals to 28.3 g/mol. In contrast, specific xylose uptake rate increased with decrease of OUR. Apparently at lower aeration, xylose is metabolized into other products rather than cell mass.

Rizzi *et al.*, 1989 and Grotjen *et al.*, 1990 found linear relation between biomass production and oxygen uptake rate for *Pichia stipitis* with Y_{cmO2} of 62.7 and 39.5 g/mol respectively. However, these studies, involving xylose fermenting yeasts, refer to ethanol as their main product, while xylitol is only a byproduct.

The influence of oxygen limitation on the conversion of xylose to xylitol as the main product and ethanol as a by product was determined. The specific xylitol and ethanol production rates increased with decrease of the specific oxygen uptake rate (Fig. 2).

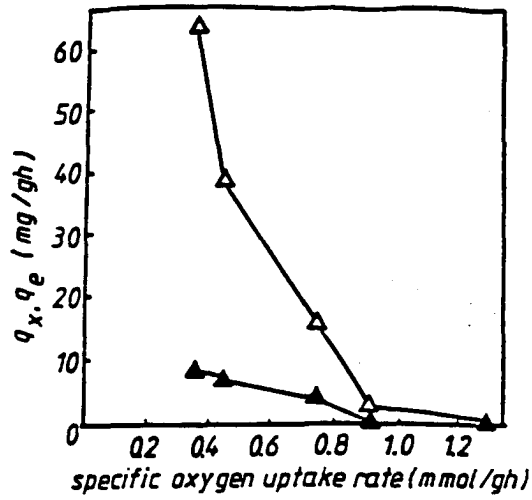

Figure 2

Specific production rates of xylitol (△) and ethanol (▲) as a function of specific oxygen uptake rate of *C. boidinii;* dilution rate. 0.05 1/h.

Upon shift to lower oxygen uptake rates, specific xylitol production rate increased more rapidly than specific ethanol production rate changing their ratio from 4, for q_{O2} = 0.74 mmol/gh to 7, for q_{O2} = 0.36 mmol/gh. This observation is consistent with our findings in batch cultures of *C. boidinii* when the xylitol to ethanol ratio increased with decreased aeration (Vandeska *et al.*, 1995a) Lower level of aeration also favour xylitol production in other xylitol producing yeasts (Furlan *et al.*, 1991, Roseiro *et al.*, 1991).

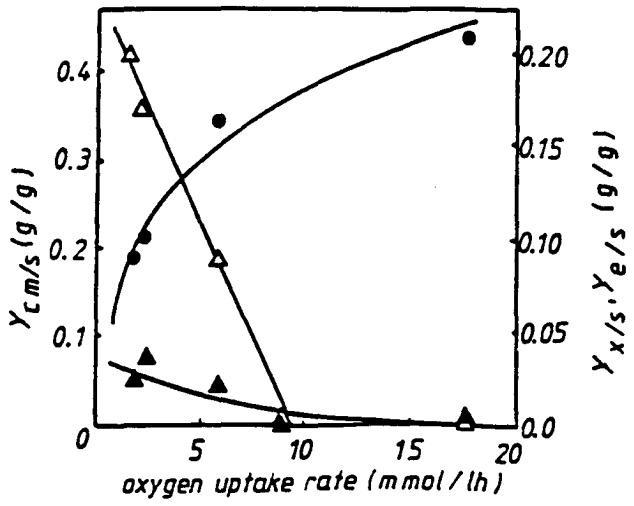

Figure 3

Yields of biomass (●), xylitol (△), and ethanol (▲) on xylose as a function of the oxygen uptake rate of *C. boidinii;* dilution rate. 0.05 1/h.

Biomass and production yields are depicted in Fig. 3 The biomass yield increased with increase of oxygen availability, while xylitol and ethanol yields have an inverse relation with oxygenation. As it can be seen from the yields, xylose metabolism is more direct towards product formation, particularly xylitol, than to cell growth as the oxygen uptake rate decreased. This observation maybe explained by the role of oxygen in xylose metabolism. Oxygen excess activates TCA cycle and so regeneration of NAD, which as a cofactor of xylitol dehydrogenase transforms xylitol into xylulose. Xylulose is further degraded through the pentose phosphate pathway and Embden-Meyerhof-Parnas pathway to give pyruvate which is entry point of TCA cycle. At OUR lower than 8,8 mmol/h, xylitol yield increased more rapidily than ethanol yield exhibiting linear relation.

The degree of oxygen limitation sets the growth rate, the substrate uptake rate and the product formation rates. The oxygen uptake rate alone can provide a reasonable estimate of the growth rate of microorganisms. The results are improved if carbon dioxide measurements are included in the calculations. Changes of the specific oxygen uptake rate as well as other process parameters in oxygen limited chemostat culture of *C. boidinii* are depicted in Table 2. It can be seen that the critical specific oxygen uptake rate for this yeast, that is the q_{O2} when xylitol excretion is triggered is 0.91 mmol/gh.

Table 2. Process parameters of oxygen limited chemostat culture of *C. boidinii*

D	Inlet gas	Agitation	DOT	q_{O2}	q_{CO2}	RQ	q_s	q_x	$Y_{x/s}$	$Y_{x/O2}$
1/h	ml/min	rev/min	%	mmol/gh	mmol/gh		mmol/gh	mmol/gh	g/g	g/g
0.052	25[a]	700	3.1	1.30	1.36	1.05	0.75	0.00	0.00	0.00
0.054	25[a]	550	2.7	0.91	1.07	1.18	1.04	0.02	0.02	0.11
0.056	20[b]	620	2.3	0.74	1.37	1.85	1.24	0.11	0.09	0.71
0.053	20[b]	400	2.1	0.44	1.89	4.30	1.60	0.26	0.17	2.90
0.056	20[b]	350	2.1	0.36	3.09	8.58	2.08	0.42	0.20	5.60

[a]Mixture of 51.9 % O_2 and N_2
[b]Air

With decrease in oxygen concentration in the inlet gas, for a constant dilution rate of 0.05 l/h the specific oxygen uptake rate decreased from 1.30 to 0.36 mmol/gh. At the same time, specific carbon dioxide production rate increased from 1.36 to 3.09 mmol/gh resulting in a respiratory quotient, RQ of 1.05 to 8.58.

Decreases in q_{O2} or increases in RQ were suggested to result in decrease in the specific rate of TCA flux and ATP production which then led to a decrease of biomass yield and increase in ethanol yield (Kuriyama and Kobayashi, 1993. Xylitol yield, likewise ethanol yield is affected in a similar manner. The difference lies in the quantity of oxygen which enhances xylitol production over ethanol (Vandeska *et al.*, 1995b).

Apart from cell mass, xylitol, ethanol and CO_2, small amounts of glycerol and ribitol were produced (0-0.25 g/l), but no detectable acetate concentrations.

CONCLUSIONS

Xylitol production by *C. boidinii* occurs under oxygen limited conditions. Partitioning between fermentation and respiration fates becomes a critical function of q_{O2} Xylitol was

produced at specific oxygen uptake rates lower than 0.91 mmol/gh. In the investigated range of oxygen uptake rates xylitol yield increased more rapidly than ethanol yield.

NOMENCLATURE

D	dilution rate (1/h)
DOT	dissolved oxygen tension (%)
m_{O2}	maintenance coefficient (mmol O_2/g cell mass h)
q_{O2}	specific oxygen uptake rate (mmol O_2/g cell mass h)
q_s	specific xylose uptake rate (g xylose/g cell mass h) or (mmol xylose/g cell mass h)
q_x	specific xylitol production rate (g xylitol/ g cell mass h) or (mmol xylitol/ g cell mass h)
q_e	specific ethanol production rate (g ethanol/ g cell mass h) or (mmol ethanol/ g cell mass h)
q_{CO2}	specific carbon dioxide production rate (mmol CO_2/ g cell mass h)
S	xylose concentration (g/l)
$Y_{cm/s}$	cell mass yield coefficient. (g cell mass/mmol xylose) or (g cell mass/ g xylose consumed)
$Y_{cm/O2}$	cell mass yield coefficient. (g cell mass/mmol O_2)
$Y_{x/s}$	xylitol yield coefficient (g xylitol/g xylose consumed)
$Y_{x/O2}$	xylitol yield coefficient (g xylitol/mmol O_2)
$Y_{e/s}$	ethanol yield coefficient (g ethanol/g xylose consumed)
OUR	oxygen uptake rate (mmol O_2/1h)
μ	specific growth rate (1/h)

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