Activities of the enzymes of the Ehrlich pathway and formation of branched-chain alcohols in *Saccharomyces cerevisiae* and *Candida utilis* grown in continuous culture on valine or ammonium as sole nitrogen source

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Valine aminotransferase, a key enzyme in both biosynthesis and breakdown of branched-chain amino acids, showed consistently higher activity in *Candida utilis* grown in continuous culture than in *Saccharomyces cerevisiae*, while pyruvate decarboxylase and alcohol dehydrogenase, the other two enzymes of the Ehrlich pathway of branched-chain alcohol formation, were lower in activity. By spheroplast lysis, it was shown that valine aminotransferase followed the distribution of pyruvate decarboxylase in being located in the cytosol. Replacement of ammonium as nitrogen source by valine during conditions of carbon or nitrogen limitation caused increased specific activities of these three enzymes in *S. cerevisiae*, but (with one exception) decreased those of *C. utilis*. Of the metabolites accumulating in the culture medium, little or no ethanol or branched-chain alcohols were present during carbon-limited growth of either organism, but the change to nitrogen limitation resulted in increases in concentration of 20- to 100-fold in pyruvate, acetate and non-pyruvate keto acids as well as the accumulation of branched-chain alcohols in both organisms, and of ethanol, ethyl acetate and glycerol in *S. cerevisiae*. When valine was the limiting nitrogen source, there was an increase in non-pyruvate keto acids and a 10- to 16-fold increase in 2-methylpropanol. Total branched-chain alcohols formed under nitrogen limitation were 2-fold higher in *S. cerevisiae* than in *C. utilis*, irrespective of nitrogen source. Accumulation of branched-chain alcohols, ethanol, acetate and glycerol was also observed during carbon-limited growth of *S. cerevisiae* with valine as nitrogen source at dilution rates above the critical rate for transition to respirofermentative growth. Less than 70% of the valine carbon metabolized during growth of *S. cerevisiae* and only 15% of that used during growth of *C. utilis* was recovered in identified metabolic products. Even allowing for losses by volatilization during aeration, this suggests that a significant amount of the valine is being metabolized by a route or routes other than the Ehrlich pathway, possibly via the action of branched-chain 2-keto acid dehydrogenase. The molar growth yield for the nitrogen source under either carbon or nitrogen limitation was significantly lower for growth on valine than for growth on ammonium, suggesting that breakdown of valine requires more energy. It is evident that not all the enzymes involved in branched-chain amino acid metabolism in yeasts have yet been identified, nor are their interactions properly understood.

Introduction

'Higher' alcohols (fusel alcohols) are the most abundant volatile flavour components in alcoholic beverages (Engan, 1981). They include propan-1-ol, 2-methylpropan-1-ol (isobutanol), (*S*)-(-)-2-methylbutan-1-ol (optically active amyl alcohol), 3-methylbutan-1-ol (isoamyl alcohol) and 2-phenylethanol (phenethyl alcohol). Acetate esters, which have even lower flavour

thresholds (MacDonald *et al.*, 1984), are also formed from these compounds. The properties and formation of both these groups have been reviewed by Berry & Watson (1987).

Fusel alcohols are formed by all the yeast species that have been examined (Guymon *et al.*, 1961 *a, b*), and are not by-products of ethanol formation. They are derived from the carbon skeletons of the corresponding amino acids by a route first postulated by Ehrlich (1907) (see also Webb & Ingraham, 1963), the enzymic basis of which was established by SentheShanmuganathan (1960). In the case of the branched-chain alcohols, this involves

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three enzymes: a branched-chain amino acid aminotransferase (EC 2.6.1.42), pyruvate decarboxylase (EC 4.1.1.1) which converts the resulting branched-chain keto acid to the branched-chain aldehyde with one carbon atom less, and alcohol dehydrogenase (EC 1.1.1.1), which catalyses the NADH-dependent reduction of this aldehyde to the corresponding primary alcohol. It has been shown for wine yeasts that the isoenzyme involved in branched-chain aldehyde reduction is the classical fermentative isoenzyme ADH-1 (Singh & Kunkee, 1977). Fusel alcohols are formed whether yeast is grown using amino acids as nitrogen source, as in wort, or with ammonium salts (Ingraham & Guymon, 1960), but the presence of glucose is essential (SentheShanmuganathan, 1960). In the first case the carbon skeletons arise from the wort amino acids, in the second case by *de novo* synthesis from glucose (see below). Fusel alcohol formation is promoted by aerobiosis (Guymon *et al.*, 1961*b*; Quain & Duffield, 1985).

Previous studies on fusel alcohol and ester formation in continuous culture (Watson & Hough, 1969; Berry & Chamberlain, 1986), have led to the suggestion that control of the production of branched-chain aldehydes lies in the supply of branched-chain keto acids (Watson & Hough, 1969). Woodward & Cirillo (1977) have shown that large amounts of these keto acids accumulate in radioactive form in the medium if ^{14}C -labelled branched-chain amino acids are administered to proline-grown *Saccharomyces cerevisiae* in the presence of glucose, though few are formed if the yeast is grown on ammonium or if glucose is absent. In the formation of branched-chain keto acids from the corresponding amino acids, one or more branched-chain amino acid aminotransferases (Bode & Birnbaum, 1988) must play a key role, and during growth on ammonium where the branched-chain keto acids are synthesized *de novo* via acetolactate or acetohydroxybutyrate, the same aminotransferase(s) will compete with pyruvate decarboxylase for the keto acids. This competition might be controlled by subcellular compartmentation (Bigelis *et al.*, 1983). No continuous culture studies have been performed on the distribution of branched-chain amino acid aminotransferase activity in yeasts.

Until recently, it was assumed (e.g. Woodward & Cirillo, 1977; Bigelis *et al.*, 1983; Large, 1986) that the Ehrlich pathway was the major, if not the only, route for the metabolic transformation of branched-chain amino acids in *S. cerevisiae*, whereas *Candida utilis* was assumed to break down the carbon skeleton completely (see references in Large, 1986). Recent work however (Dickinson & Dawes, 1992) has shown that the branched-chain 2-keto acid dehydrogenase multienzyme system (EC 1.2.4.4) found in bacteria (Massey *et al.*, 1976) is also present in *S. cerevisiae*. Any work on fusel alcohol

formation thus needs to address the problem of the possible decarboxylation of metabolites of branched-chain amino acids via more than one route.

The objectives of the present work were to examine the effect of changes in the glucose concentration, the nitrogen source and the growth rate on (1) the activities and subcellular location of the three enzymes of the Ehrlich pathway (and a few other key enzymes) in two yeast species, *S. cerevisiae* and *C. utilis*, growing in aerobic continuous culture on defined medium, and (2) the steady-state concentrations of 2-methylpropanol, 2-methylbutanol and 3-methylbutanol, as well as several other metabolic products. This permits for the first time a direct comparison under identical growth conditions of these parameters for two yeasts of different physiological type, Crabtree-positive and Crabtree-negative (Alexander & Jeffries, 1990).

Methods

Micro-organisms and growth conditions. *Saccharomyces cerevisiae* CBS 8066 and *C. utilis* CBS 621 were maintained on glucose (0.5%)/yeast extract (0.5%)/malt extract (2%, w/v)/agar (2%, w/v) slopes. They were grown aerobically on defined medium in chemostat cultures (working volume 750 ml) at 30 °C, at a dilution rate of 0.1 h⁻¹ unless otherwise stated, with the pH controlled to 5.0 by automatic addition of 1 M-NaOH. The stirrer speed was 1000 r.p.m. and the gas flow rate was 600 ml air min⁻¹. The medium was that of Bruinenberg *et al.* (1983) except that Bevaloid 6560 defoamer (Bevaloid Ltd) was used. The carbon source was glucose. The oxygen tension was monitored with an autoclavable galvanic oxygen electrode and maintained between 70 and 100% of air saturation. For glucose-limited growth, the concentration was 27.8 mM (5 g l⁻¹), and for nitrogen-limited growth, 278 mM (50 g l⁻¹). The nitrogen source was either ammonium sulphate (10 mM) or valine (20 mM). The medium was sterilized by filtration through a Sartorius Sartobran-PH filter cartridge (pore size combination: 0.45/0.2 µm). Growth was measured as optical density at 540 nm and converted to dry weight using a calibration curve for each organism. Samples were removed from the vessel in screw-top bottles sealed by a rubber liner, centrifuged at 5 °C, and the supernatants stored in screw-top bottles at -18 °C.

Harvesting of cells and preparation of cell-free extracts. Cells were centrifuged at 16000 g at 5 °C, washed in water, and used immediately for cell fractionation work. Otherwise, they were stored at -18 °C until required for enzyme assays. Extracts were prepared by suspending the cells in 2 vols 150 mM-potassium phosphate buffer, pH 6.5 containing 5 mM-MgSO₄, 1 mM-thiamin pyrophosphate and 2 mM-dithiothreitol (modified from Van Urk *et al.*, 1989) and passing three times through a French pressure cell at 4 °C. The resulting material was centrifuged for 15 min at 40000 g at 5 °C.

Preparation of spheroplasts. For *S. cerevisiae*, 1 g wet wt of cells was preincubated in 5 ml 2.5 mM-dithiothreitol in 0.1 M-Tris/HCl, pH 9 at 28 °C for 20 min (Skoneczny *et al.*, 1988). Cells were then pelleted (12000 g, 10 min), washed with spheroplast buffer A (2 M-sorbitol, 1 mM-EDTA in 5 mM-MOPS, pH 7.2) and resuspended in 2 ml of buffer A containing 1% (v/v) 2-mercaptoethanol and 2 mg Lyticase (Sigma) (Scott & Schekman, 1980) (g wet wt of cells)⁻¹. The suspension was incubated at 30 °C and spheroplast formation monitored (as described by Bruinenberg *et al.*, 1985) until 50–70% of the cells had become spheroplasts, or non-osmotic lysis was evident. Spheroplasts of *C. utilis* were prepared by the method of Bruinenberg *et al.* (1985). Cells were

harvested and washed with spheroplast buffer B (2 M-sorbitol, 1 mM-EDTA, 1 mM-MgCl₂ in 25 mM-potassium phosphate, pH 7.5) and resuspended in 2 ml of buffer B containing 2 mg Lyticase (g wet wt of cells)⁻¹. The suspension was incubated at 30 °C and spheroplast formation monitored. With both organisms, the spheroplasts were harvested by centrifugation at 3000 g for 10 min at 5 °C, washed twice and resuspended in 10 ml of appropriate spheroplast buffer.

Lysis of spheroplasts and isolation of subcellular fractions. Spheroplasts were lysed by gentle osmotic disruption, based on method IV of Bruinenberg *et al.* (1985) in which samples (10 ml) were dialysed at 15 °C against 5 litres of spheroplast buffer not containing sorbitol and the decrease in sorbitol concentration was monitored with a refractometer until the critical concentration was reached at which cell lysis was maximal and organelle lysis had not quite begun (0.9 M for *S. cerevisiae* and 0.7 M for *C. utilis*). Dialysis was then stopped and whole cells and unbroken spheroplasts were removed by two centrifugations at 1930 g for 15 min at 5 °C. The supernatant (T) was then centrifuged at 12000 g for 10 min to provide pellet P₁ and the supernatant subjected to 45000 g for 20 min to provide P₂ and a final supernatant S. P₁ and P₂ were washed and resuspended in 1 vol. of spheroplast buffer containing the appropriate sorbitol concentrations. All samples were stored at -15 °C and enzyme activity measured within 2 weeks.

Enzyme assays. Enzyme assays on samples from steady-state cultures are averages of duplicate determinations. The error in most assay rate measurements was $\pm 10\%$. Assays were all performed in 1 ml reaction volumes at 25 °C. One unit of enzyme activity is the amount required to catalyse the transformation of 1 μ mol of substrate or the formation of 1 μ mol of product min⁻¹. Assays were performed according to the following methods: alcohol dehydrogenase (McGill & Dawes, 1971), pyruvate decarboxylase and citrate synthase (Clarkson *et al.*, 1991), alkaline phosphatase (Green & Large, 1984), NAD- and NADP-dependent glutamate dehydrogenases (Doherty, 1970), glucose-6-phosphate dehydrogenase (Bergmeyer, 1963; modified by the use of pH 8 and with the addition of 0.83 units of 6-phosphogluconate dehydrogenase per ml of reaction mixture). Valine aminotransferase was assayed as follows (based on Large & Robertson, 1988): 20 mM-sodium 2-oxoglutarate was incubated with 0.1 mM-pyridoxal phosphate, 50 mM-potassium phosphate buffer, pH 7.8, extract and water (final volume, 1 ml). The reaction was started by addition of 20 mM-L-valine, and stopped after 10 min by heating for 2 min at 100 °C. Samples were removed and the glutamate measured with Boehringer test-combination 139092. The activity was calculated from the difference in glutamate between 10 min and zero time samples.

Chemical determinations. All medium analyses were based on duplicate determinations. Samples were thawed slowly at 5 °C, still in sealed bottles. Pyruvate was measured enzymically from the change in absorbance at 340 nm when 0.2 mM-NADH, 120 mM-triethanolamine/HCl, pH 7.6, diluted sample and 9.1 units of rabbit muscle lactate dehydrogenase (LDH) (Sigma) were incubated together. Total keto acids were measured as 2,4-dinitrophenylhydrazones (Aki & Ichihara, 1970), and non-pyruvate keto acids were obtained by subtraction of the pyruvate concentration. Acetate was measured using Boehringer food analysis kit 148261. Since samples for glycerol determination contained high concentrations of pyruvate, the latter was removed by converting it to lactate by incubating 0.5 ml of diluted sample with 1 μ mol NADH and 18 units of LDH in 0.2 M-triethanolamine/HCl buffer, pH 7.6 in a total volume of 1 ml at 25 °C for 30 min. The LDH was then inactivated by heating for 2 min at 100 °C. After cooling, the glycerol content of the sample was determined with Boehringer test-combination 148270. Ethanol and glucose were determined as described by Clarkson *et al.* (1991). Branched-chain alcohols and ethyl acetate were extracted from the growth medium into three successive equal volumes of diethyl ether after addition of butan-2-ol (2.75 mM) as internal standard. After concentration of the combined ethereal extracts,

samples were submitted to GLC on a Supelco Carbowax C/0.2% Carbowax 1500 column (1.5 m $\times$ 4 mm) (Supelchem UK Ltd) (injection port and detector temperatures, 200 °C; column temperature, 115 °C), with N₂ carrier gas (flow rate 30 ml min⁻¹) with flame-ionization detection and quantified by comparison of peak area with a standard curve for butan-2-ol. Peaks were identified by comparison of their retention times with authentic standards, and in some cases confirmed by GLC-MS. Ammonium was measured by the indophenol method (Chaney & Marbach, 1962), and valine with 2,4,6-trinitro-benzenesulphonic acid (Satake *et al.*, 1960). Protein was determined by the Bradford (1976) method.

Results and Discussion

Saccharomyces cerevisiae and *Candida utilis* were grown in defined medium in aerobic continuous culture at glucose concentrations of 27.8 mM (carbon-limiting) and 278 mM (nitrogen-limiting) with either 20 mM-ammonium or 20 mM-valine as sole nitrogen source. The cells were examined for the activities and subcellular distribution of enzymes of the Ehrlich pathway, and the biomass, substrate and product concentrations of the medium were measured.

Specific activities of enzymes of the Ehrlich pathway and other key enzymes in crude extracts during growth on ammonium as nitrogen source

In *S. cerevisiae*, with ammonium as nitrogen source, the change to nitrogen limitation, which induces respiro-fermentative growth (ethanol formation under aerobic growth conditions; Alexander & Jeffries, 1990), produced increases in the specific activities of valine aminotransferase and pyruvate decarboxylase, compared with glucose-limited growth, whereas NADP-linked glutamate dehydrogenase was unchanged and alcohol dehydrogenase, NAD-linked glutamate dehydrogenase and glucose-6-phosphate dehydrogenase decreased (Table 1). In *C. utilis*, although respirofermentative growth does not occur at high glucose concentrations, the effect of the change to nitrogen limitation on enzyme activities was similar, except that NADP-linked glutamate dehydrogenase and valine aminotransferase were decreased and, of the other enzymes of the Ehrlich pathway, the specific activity of pyruvate decarboxylase was elevated and alcohol dehydrogenase was unchanged. With the exception of these last two enzymes, enzyme specific activities in *C. utilis* were much higher than in *S. cerevisiae* under all the growth conditions shown in Table 1.

Specific activities of enzymes of the Ehrlich pathway and other key enzymes in crude extracts during growth on valine as nitrogen source

During growth with valine as nitrogen source, all the enzymes of the Ehrlich pathway were elevated in *S.*

Table 1. Specific activities of enzymes of the Ehrlich pathway and other key enzymes during growth of *Saccharomyces cerevisiae* and *Candida utilis* in continuous culture under carbon or nitrogen limitation

Enzyme	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]			
	Ammonium as nitrogen source		Valine as nitrogen source	
	Carbon limitation	Nitrogen limitation	Carbon limitation	Nitrogen limitation
<i>(a) Saccharomyces cerevisiae</i>				
Valine aminotransferase	29	93	682	122
Pyruvate decarboxylase	180	290	372	459
Alcohol dehydrogenase	597	207	699	248
Glutamate dehydrogenase (NAD)	20	6	101	76
Glutamate dehydrogenase (NADP)	965	995	511	323
Glucose-6-phosphate dehydrogenase	93	45	163	72
<i>(b) Candida utilis</i>				
Valine aminotransferase	1840	747	1620	2740
Pyruvate decarboxylase	42	204	31	106
Alcohol dehydrogenase	235	283	22	8
Glutamate dehydrogenase (NAD)	103	28	1450	256
Glutamate dehydrogenase (NADP)	3000	1300	458	365
Glucose-6-phosphate dehydrogenase	1520	826	1130	993

Table 2. Subcellular distribution in pellets (P_1 and P_2) and 45 000 g supernatant (S) of enzymes of the Ehrlich pathway in *Saccharomyces cerevisiae* and marker enzymes

Data are from a representative spheroplast preparation. Essentially similar figures were obtained on other preparations from both organisms. The first column is units of enzyme activity or mg protein in the total spheroplast lysate. The second column is $100 \times [P_1 + P_2 / (P_1 + P_2 + S)]$, the third column is $100 \times [S / (P_1 + P_2 + S)]$ and the fourth column $100 \times [(P_1 + P_2 + S) / T]$, where P_1 , S etc., are the total activities in the respective fractions.

Enzyme	Carbon-limited				Nitrogen-limited			
	Total activity (T)	$P_1 + P_2$ (%)	S (%)	Recovery (%)	Total activity (T)	$P_1 + P_2$ (%)	S (%)	Recovery (%)
<i>Ammonium as nitrogen source</i>								
Citrate synthase	0.9	96	4	121	0.2	51	49	113
Alkaline phosphatase	0.4	11	89	58	2.3	4	96	82
Valine aminotransferase	2.5	12	88	89	3.3	5	95	100
Pyruvate decarboxylase	15.6	1	99	125	15.3	13	87	125
Alcohol dehydrogenase	50.6	3	97	115	7.2	5	95	42
Protein	84.6	6	94	105	34.9	4	96	100
<i>Valine as nitrogen source</i>								
Citrate synthase	6.5	55	45	77	1.3	37	63	93
Alkaline phosphatase	1.9	9	91	64	2.4	7	93	80
Valine aminotransferase	34.9	4	96	64	8.3	4	96	56
Pyruvate decarboxylase	8.6	3	97	70	31.2	0	100	77
Alcohol dehydrogenase	30.1	3	97	59	16.9	1	90	91
Protein	50.0	7	93	99	67.9	3	97	79

cerevisiae. Nitrogen limitation led to lower valine aminotransferase activity in *S. cerevisiae* compared with carbon-limited conditions but in *C. utilis* it was higher. In the latter organism, alcohol dehydrogenase and NADP-linked glutamate dehydrogenase were much lower under both growth conditions when valine was the

nitrogen source (Table 1). The latter would be expected, because the enzyme will have no biosynthetic role during growth on valine. NAD-linked glutamate dehydrogenase in contrast was elevated in both yeasts under these conditions, as would be expected in view of its known role (Miller & Magasanik, 1990) of releasing ammonia

Table 3. Subcellular distribution in pellets (P_1 and P_2) and 45000 g supernatant (S) of enzymes of the Ehrlich pathway in *Candida utilis* and marker enzymes

See legend to Table 2.

Enzyme	Carbon-limited				Nitrogen-limited			
	Total activity (T) in crude	$P_1 + P_2$ (%)	S (%)	Recovery (%)	Total activity (T) in crude	$P_1 + P_2$ (%)	S (%)	Recovery (%)
<i>Ammonium as nitrogen source</i>								
Citrate synthase	11.6	23	77	40	15.6	23	77	88
Alkaline phosphatase	2.5	8	92	74	10.3	1	99	101
Valine aminotransferase	105.5	3	97	86	90.0	2	98	76
Pyruvate decarboxylase	2.4	9	91	132	24.6	1	99	93
Alcohol dehydrogenase	13.6	25	75	79	34.1	3	97	88
Protein	57.6	11	89	66	120.3	6	94	76
<i>Valine as nitrogen source</i>								
Citrate synthase	72.0	3	97	88	6.8	13	87	88
Alkaline phosphatase	1.1	5	95	70	18.2	2	98	57
Valine aminotransferase	91.0	8	92	121	141.9	4	96	82
Pyruvate decarboxylase	1.7	ND	ND	ND	5.5	0	100	77
Alcohol dehydrogenase	1.2	ND	ND	ND	0.4	ND	ND	ND
Protein	56.2	6	94	120	51.9	2	98	68

ND, Not determined.

from glutamate (formed by the action of valine aminotransferase). Similar results for this enzyme were obtained by Zwart *et al.* (1983) during growth of *C. utilis* in continuous culture on L-alanine. The recorded levels of pyruvate decarboxylase in Table 1 are in good agreement with the data of Van Urk *et al.* (1989), but our alcohol dehydrogenase activities are lower, due to different assay conditions. The levels of aminotransferase activity are adequate to account for the observed levels of non-pyruvate keto acids.

Subcellular location of the enzymes of the Ehrlich pathway during different growth conditions

It is known that the branched-chain amino acid aminotransferase of mammalian tissue varies in its subcellular location according to the metabolic role of the tissue, so that when the enzyme has a biosynthetic role, e.g. in brain or white muscle, it is located in the cytosol, and when it has a degradative role, e.g. in heart, it is located in the mitochondria (Hutson, 1988). In *Neurospora crassa*, separate branched-chain amino acid aminotransferases (with slightly different substrate specificities) exist in cytosol and mitochondria, with the cytosolic enzyme being thought to be involved in amino acid breakdown and the mitochondrial enzyme in biosynthesis (Collins & Wagner, 1973). In yeast, the enzymes of isoleucine and valine biosynthesis are known to be mitochondrial (Ryan & Kohlhaw, 1974), but nothing is known of the subcellular location of the

enzymes of the Ehrlich pathway (Bigelis *et al.*, 1983). Ryan *et al.* (1973) obtained evidence that the glutamate:2-oxoisocaproate aminotransferase activity of baker's yeast was located in both cytosolic and particulate cell fractions.

It thus seemed plausible that the subcellular distribution of branched-chain amino acid aminotransferase might change between conditions in which it has a degradative role such as growth on valine as the sole nitrogen source, and conditions in which it has a biosynthetic role, such as when ammonium serves as sole nitrogen source. Tables 2 and 3 report the distribution of the three enzymes of the Ehrlich pathway, the mitochondrial marker enzyme citrate synthase and the non-sedimenting marker enzyme, alkaline phosphatase (Jenkins *et al.*, 1983) during the various growth conditions. Pyruvate decarboxylase was found to be almost exclusively located in the cytosol in both organisms, in agreement with the work of Van Urk *et al.* (1989). Under all growth conditions and with both organisms, the distribution of valine aminotransferase closely followed that of alkaline phosphatase and pyruvate decarboxylase, leading us to conclude that it also was cytosolic (not sedimentable), and thus did not change its subcellular location under the different conditions studied, as suggested by Bigelis *et al.* (1983). It should be noted, however, that for certain of our spheroplast preparations, especially of *C. utilis*, the behaviour of the mitochondrial marker enzyme citrate synthase suggests that there has been significant lysis of the mitochondria.

Table 4. Growth yields and steady-state concentrations of substrates and metabolites detected in the culture supernatants during growth of *Saccharomyces cerevisiae* in continuous culture under carbon or nitrogen limitation

Substrate or metabolite	Concentration (mM)			
	Ammonium as nitrogen source		Valine as nitrogen source	
	Carbon limitation	Nitrogen limitation	Carbon limitation	Nitrogen limitation
Culture OD ₅₄₀	6.2	10.0	5.6	9.2
Dry wt (mg ml ⁻¹)	2.28	3.88	2.03	3.54
<i>Substrates</i>				
Glucose in inflowing medium	26.0	235.0	25.2	218.0
Glucose in culture supernatant	0	10.8	0	9.5
Nitrogen source in inflowing medium	20.0	20.0	24.3	21.1
Nitrogen source in culture supernatant	9.1	0	9.5	0
Glucose metabolized (%)	100	96	100	96
Nitrogen source metabolized (%)	54	100	60	100
<i>Molar growth yields</i> [g dry wt (mol glucose or g-atom nitrogen) ⁻¹]				
Glucose	88	17	81	17
Nitrogen source	209	194	137	168
<i>Metabolites</i>				
Pyruvate	0.04	3.68	0.11	9.80
Other keto acids	0.14	3.21	1.56	5.00
Acetate	0.01	1.68	0.05	1.92
Ethyl acetate	0	0.70	0	7.90
Glycerol	0.04	2.26	0.09	1.70
Ethanol	0	150.0	0	158.0
2-Methylpropanol	0	0.45	0	7.30
2-Methylbutanol	0	0.06	0	0.31
3-Methylbutanol	0	0.20	0	0.38

The data for valine aminotransferase do not agree with the results of Ryan *et al.* (1973) for leucine aminotransferase in baker's yeast, where activity was found both in particulate (80%) and supernatant (20%) fractions of spheroplast lysates. It is possible that valine and leucine are metabolized by different aminotransferases, although there is some evidence to suggest that *S. cerevisiae* only contains a single aminotransferase for branched-chain amino acids (Dickinson & Dawes, 1992). An alternative explanation may lie in differences of strain or growth conditions.

The presence of pyruvate decarboxylase and valine aminotransferase in the same subcellular compartment removes the possibility of control of biosynthesis and degradation of L-valine by subcellular compartmentation (Bigelis *et al.*, 1983). In *de novo* biosynthesis of valine, the two enzymes will compete directly for 2-oxoisovalerate, and the most likely possible control is thus kinetic. No K_m values for branched-chain keto acids seem to have been reported, either for pyruvate decarboxylase or for any branched-chain amino acid aminotransferase (Ichihara, 1985), so it is not possible to predict the outcome of such competition. Our conclusion also implies that a transport mechanism must exist to allow *de novo* synthesized branched-chain keto acids to leave the

mitochondria where they have been made (Ryan & Kohlhaw, 1974).

Steady-state concentrations of products accumulated in the medium during continuous culture of S. cerevisiae

The shift to nitrogen limitation increased steady-state biomass concentration by 70%, irrespective of nitrogen source (Table 4). It also caused the accumulation of ethanol, absent from the carbon-limited growth medium, at significant concentrations [150 mM, equivalent to 0.7% (w/v)], so lowering the growth yield considerably, and produced a 100-fold increase in the concentration of pyruvate, irrespective of the nitrogen source. Less spectacular, but equally significant increases were observed in the steady-state concentrations of glycerol, acetate and ethyl acetate. The molar growth yields during nitrogen limitation were 194 g dry wt (g-atom nitrogen)⁻¹ for growth on ammonium and 168 g dry wt (g-atom nitrogen)⁻¹ for growth on valine. During respirofermentative metabolism 96% of the glucose was consumed, of which 23% was recovered as ethanol. Nitrogen limitation stimulated the steady-state concentration of non-pyruvate keto acids (assumed to be substantially 2-oxoisovalerate) by 24-fold during growth

Table 5. Growth yields and steady-state concentrations of substrates and metabolites detected in the culture supernatants during growth of *Candida utilis* in continuous culture under carbon or nitrogen limitation

Substrate or metabolite	Concentration (mM)			
	Ammonium as nitrogen source		Valine as nitrogen source	
	Carbon limitation	Nitrogen limitation	Carbon limitation	Nitrogen limitation
Culture OD ₅₄₀	5.5	12.4	4.5	9.4
Dry wt (mg ml ⁻¹)	1.60	3.23	1.18	3.00
<i>Substrates</i>				
Glucose in inflowing medium	27.2	235	35	272
Glucose in culture supernatant	0	165.5	0	192
Nitrogen source in inflowing medium	19.8	18.5	22.1	21.4
Nitrogen source in culture supernatant	4.4	0	9.1	0
Glucose metabolized (%)	100	30	100	30
Nitrogen source metabolized (%)	78	100	59	100
<i>Molar growth yields</i> [g dry wt (mol glucose or g-atom nitrogen) ⁻¹]				
Glucose	59	47	34	38
Nitrogen source	104	175	91	140
<i>Metabolites</i>				
Pyruvate	0.06	3.30	0.11	2.81
Other keto acids	0	9.30	0.30	6.10
Acetate	0	2.26	0.04	2.20
Glycerol	0	0.12	0	0.06
Ethanol	0	0.60	0	3.08
2-Methylpropanol	0	0.30	0.32	3.01
2-Methylbutanol	0	0	0	0.32
3-Methylbutanol	0	0	0	0.19

on ammonium and by 3-fold during growth on valine, as would be expected from the observations of Woodward & Cirillo (1977). The corresponding data for the fusel alcohols shows that none were detectable if glucose was limiting in the culture, but that when glucose was in excess, i.e. under nitrogen limitation (Äyräpää, 1971), 2-methylpropanol was the most important alcohol formed (as also observed during aerated growth by Guymon *et al.*, 1961*b*). More branched-chain alcohols were present when valine was the nitrogen source rather than ammonium, confirming the data of Watson & Hough (1969) with leucine. The largest increase (16-fold) of the fusel alcohols is in 2-methylpropanol, as would be expected from its close relationship to valine, but 2-methylbutanol and 3-methylbutanol were also increased (5.5-fold and 1.6-fold respectively).

Steady-state concentrations of products accumulated in the medium during continuous culture of C. utilis

In *C. utilis* grown under nitrogen limitation the steady-state pyruvate concentration was increased 28- to 55-fold (Table 5), and the increases in non-pyruvate keto acids were higher than in *S. cerevisiae*. The molar growth yields for nitrogen-limited growth were 175 g dry wt (g-

atom nitrogen)⁻¹ for growth on ammonium and 140 for growth on valine. The lack of respirofermentative metabolism, typical of Crabtree-negative yeasts, means that only small quantities of ethanol were formed under nitrogen limitation, and only 30% of the glucose was consumed, irrespective of nitrogen source. Glycerol and ethyl acetate accumulation were insignificant. Acetate however accumulated to an even greater extent than in *S. cerevisiae*. The quantities and relative proportions of the three branched-chain alcohols were different in *C. utilis*, and the total amounts formed during steady-state growth under nitrogen limitation were about half those formed by *S. cerevisiae*. 2-Methylpropanol was detectable even in carbon-limited cells when valine was the nitrogen source, and in nitrogen-limited cells it was increased 10-fold by valine. The methylbutanols were only found in nitrogen-limited growth on valine.

Effect of varying the growth rate on the accumulation of metabolites in the culture medium of S. cerevisiae growing on valine as sole nitrogen source under carbon limitation

During carbon-limited growth, at low growth rates, respiratory metabolism occurs, but when a critical

Fig. 1. Effect of dilution rate on the steady rate concentrations of substrates and fermentation products during growth of *Saccharomyces cerevisiae* on limiting glucose with L-valine as the nitrogen source. (a) $\blacktriangle$, Cell yield [g (g glucose) $^{-1}$]; $\bullet$, percentage of glucose metabolized; $\circ$, percentage of valine metabolized; (b) concentrations of metabolites in the medium: $\circ$, ethanol; $\bullet$, glycerol; $\square$, pyruvate; $\blacktriangle$, acetate; (c) $\blacktriangledown$, 2-methylpropanol; $\triangle$, non-pyruvate keto acids.

dilution rate is exceeded, ethanol production is triggered, despite the aerobic conditions (respirofermentative growth), and this is accompanied by a drastic drop in the

biomass produced (Fiechter *et al.*, 1981; Postma *et al.*, 1989). This approach enables branched-chain alcohol production to be studied under conditions in which glucose catabolite repression is minimized. The effect of growth rate on valine metabolism in *S. cerevisiae* was investigated by analysis of culture supernatants obtained from steady-state cultures with dilution rates in the range 0.07–0.3 h^{-1} at 27.8 mM-glucose and 20 mM-valine. Growth was carbon-limited up to $D = 0.3 \text{ h}^{-1}$ (Fig. 1a), where growth became limited by a medium component other than carbon or nitrogen, possibly sodium molybdate (Postma *et al.*, 1989). Up to $D = 0.2 \text{ h}^{-1}$, cell yield and the percentage of glucose and valine metabolized remained constant (Fig. 1a) and the concentration of pyruvate in the medium increased (Fig. 1b); above this dilution rate, they decreased. The onset of ethanol and acetate production (Fig. 1b) and therefore of respirofermentative growth coincided with the decrease in biomass production observed above $D = 0.2 \text{ h}^{-1}$. The metabolites formed were lower in concentration than those observed in the work of Postma *et al.* (1989), in which ammonium was the nitrogen source, but the only significant difference that could be ascribed to a contribution from the carbon skeleton of valine was an increase in the production of non-pyruvate keto acids (assumed to be predominantly 2-oxoisovalerate) and a decrease in the biomass yield. Below the critical dilution rate of 0.2 h^{-1} , the non-pyruvate keto acids increased with increasing growth rate, both as concentration in the supernatant (Fig. 1c) and also as proportion of the metabolized valine, but above 0.2 h^{-1} , they decreased along with the valine used, partly due to conversion to 2-methylpropanol. The latter was only observed during respirofermentative growth and it increased in concentration with growth rate up to $D = 0.24 \text{ h}^{-1}$ before beginning to fall (Fig. 1c), although its production was constant relative to valine metabolized and biomass produced at all growth rates above 0.2 h^{-1} . No methylbutanols were detected. Our observations are essentially in agreement with those of Postma *et al.* (1989). Our lower critical dilution rate (0.2 h^{-1} as against 0.38 h^{-1}) and the lower concentrations of metabolites produced cannot be due to the lower glucose concentration used in the present study (Rieger *et al.*, 1983). It seems probable that they are due to the higher energy requirement when valine is used as nitrogen source. However, our data are supported by recent work by Larsson *et al.* (1993), using *S. cerevisiae* CBS 246, who observed an identical critical dilution rate for transition to respirofermentative growth of 0.2 h^{-1} during glucose-limited growth at high ammonium concentrations. The decrease in glycerol formed at dilution rates above 0.24 h^{-1} (Fig. 1b) may relate to the increase in branched-chain alcohols (Quain & Duffield, 1985).

Relationship of the measured enzyme activities to the concentration of branched-chain alcohols formed

It can be calculated from the enzyme activities in Table 1 and the rate of biomass synthesis (assuming a 50% protein content), that the rate of synthesis of all the three enzymes of the Ehrlich pathway is in all the conditions studied and in both organisms more than sufficient to account for the concentration of branched-chain alcohols formed. It seems unlikely that the activity of valine aminotransferase is a controlling element, since in *C. utilis* where it is much more active, the concentrations of fusel alcohols accumulating are actually lower than in *S. cerevisiae*. It is clear that despite the significant differences in enzyme specific activities between the two yeasts, the differences in the concentration of fusel alcohols between the two species *S. cerevisiae* and *C. utilis* cannot be explained on this basis.

Fate of valine as nitrogen source

The decreased molar biomass yield in both organisms during growth on valine suggests that the carbon skeleton of valine is not contributing to cell biomass, and also that the assimilation of valine nitrogen places an additional energy requirement on the cell. In *S. cerevisiae*, the 2-methylpropanol formed during nitrogen-limited growth only accounts for 37% of the carbon of the valine that has been used. Even if it is assumed that all the additional pyruvate and other keto acids formed at high glucose concentration also arise from valine, more than 30% of the valine consumed is still unaccounted for, possibly as metabolites not measured, since the cellular carbon has not increased. One possible explanation of this is that at least some of the valine used has been metabolized via some other metabolic route. Such a route might be the branched-chain 2-keto acid dehydrogenase system recently described in *S. cerevisiae* by Dickinson & Dawes (1992). We have found that this enzyme system is indeed present, albeit at rather low activity, in *S. cerevisiae* but not *C. utilis*, grown in batch culture on the carbon-limiting medium (C. Wadforth & P. J. Large, unpublished data). The suggestion is supported by the 23-fold increase in specific activity of branched-chain amino acid aminotransferase during carbon-limited growth on valine (Table 1). In *C. utilis*, the branched-chain 2-keto acid dehydrogenase pathway, if present, might account for an even greater proportion of the valine metabolized, since in *C. utilis* 85% of the valine carbon is not accounted for. However, since no special precautions were taken to prevent volatile metabolites being stripped from the medium by the aerating air, it is not possible to exclude losses by volatilization as contributing to the poor recoveries of these metabolites (although the ethanol

would be more significantly affected than the branched-chain alcohols). More data will be necessary to establish for certain whether or not valine contributes to biomass cell build-up. Further work using [¹⁴C]valine and kinetic studies of the aminotransferase, decarboxylase and branched-chain keto acid dehydrogenase will also be necessary to establish for certain the relative contribution of different metabolic routes to the fate of valine.

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