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Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium

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Abstract Supplementation of a chemically defined medium with amino acids or succinate to improve heterologous xylanase production by a prototrophic *Saccharomyces cerevisiae* transformant was investigated. The corresponding xylanase production during growth on ethanol in batch culture and in glucose-limited chemostat culture were quantified, as the native *ADH2* promoter regulating xylanase expression was derepressed under these conditions. The addition of a balanced mixture of the preferred amino acids, Ala, Arg, Asn, Glu, Gln and Gly, improved both biomass and xylanase production, whereas several other individual amino acids inhibited biomass and/or xylanase production. Heterologous protein production by the recombinant yeast was also improved by supplementing the medium with succinate. The production of heterologous xylanase during growth on ethanol or glucose could thus be improved by supplementing metabolic precursors in the carbon- or nitrogen-metabolism.

Introduction

Cultivation of recombinant yeast in defined media for heterologous protein production is advantageous due to easier quantification of growth requirements, simpler purification of the product and the propensity towards rapid scale-up (Greasham and Herber 1997). In the present study, the possible limitation of heterologous protein production in recombinant *Saccharomyces cerevisiae* by an insufficient availability of amino acids or succinate during growth in defined medium was investigated. A prototrophic recombinant *S. cerevisiae* strain producing a fungal xylanase under the transcriptional control of the *ADH2* promoter was used as a model system. Compared to production levels in complex medium, very low production levels of heterologous xylanases by auxotrophic recombinant *S. cerevisiae* in defined media have been reported (Donald et al. 1994; Pérez-González et al. 1996; La Grange et al. 1996; Nuyens et al. 2001; our unpublished results). In an auxotrophic recombinant *S. cerevisiae* strain, heterologous xylanase production improved dramatically by supplementation of the defined medium with an excess of amino acids (Görgens et al. 2004). An apparent limitation in the availability of amino acid building blocks for heterologous xylanase production by auxotrophic yeasts thus existed. In the present study, a prototrophic strain was used to investigate the presence of a similar limitation for amino acids or succinate for heterologous xylanase production. The defined medium was supplemented during growth on ethanol in batch culture or during glucose-limited growth in chemostats, when the native *ADH2* promoter, used to regulate xylanase expression, was derepressed.

Supplementation of defined media with exogenous nitrogen sources has previously improved biomass formation by *S. cerevisiae*, especially during fully respiratory growth on ethanol (Chen et al. 1993; Gu et al. 1991; Thomas and Ingledew 1990, 1992). Nitrogen sources most strongly preferred by *S. cerevisiae* include glutamine, asparagine and ammonium. These compounds are utilised first from mixtures of nitrogen sources and support higher growth rates than less preferred nitrogen sources (Ter Schure et al. 2000; Dubois and Messenguy 1997; Wiame et al. 1985; Cooper 1982). Exogenous amino acids can be incorporated directly into biomass during consumption (Albers et al. 1996), whilst amino acids have been known to improve the production of heterologous proteins by *S. cerevisiae* in defined media (Mendoza-Vega et al. 1994;

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Wittrup and Benig, 1994; Toman et al. 2000; Blechl et al. 1992). The proteolytic degradation of heterologous protein products may be decreased by supplementation of the medium with pure amino acids, such as arginine and lysine (Choi et al. 2000; Kang et al. 2000; Chung and Park 1998), or complex mixtures, such as casamino acids (Coppella and Dhurjati 1989; Boze et al. 2001; Werten et al. 1999; Goodrick et al. 2001; Sreekrishna et al. 1997). Metabolite balancing during recombinant protein production has identified a depletion of amino acids and biosynthetic precursors from the TCA cycle during recombinant protein production (Jin et al. 1997). In the present investigation, medium supplementation was aimed at increasing the availability of amino acid building blocks for biosynthesis or stimulating the TCA cycle by succinate addition, during heterologous xylanase production by a prototrophic *S. cerevisiae* transformant growing on ethanol or glucose.

Materials and methods

Strains and plasmids

An auxotrophic, recombinant strain of *S. cerevisiae* Y294 [*pDLG5*] with genotype [*ura3*/*URA3*, *fur1*::*LEU2*, *trp1*, *his3*], producing heterologous β-1,4-xylanase by expression of a plasmid-based *XYN2* gene under the control of the native *ADH2*-promoter, was constructed previously (La Grange et al. 1996). In the present study, a prototrophic variant of this strain, *S. cerevisiae* Y294 [*ura3*/*URA3*, *fur1*::*LEU2*, *trp1*::*TRP1*, *his3*::*HIS3*, *pDLG5*], was created by replacement of the *trp1*, *his3* alleles with wild-type *TRP1* and *HIS3* alleles using Rothstein replacement (Rothstein 1991). This latter prototrophic strain was used throughout the present investigation. Xylanase production was regulated by the native *ADH2* promoter, which is derepressed by the diauxic shift to growth on ethanol.

Cultivation of recombinant yeast

Medium supplements were first screened individually in baffled shake-flask cultures, prior to quantification in batch culture. The prototrophic transformant was cultivated in a defined minimal medium (Verduyn et al. 1992) containing 20 g l^{-1} glucose as the carbon source. Batch and continuous fermentation in the defined medium were performed as previously described (Görgens et al. 2001). In batch cultures, amino acid and succinate supplements were added to cultures just before the shift from growth on glucose to growth on ethanol, resulting in maximal availability during the heterologous xylanase production phase. Xylanase production was regulated by the native *ADH2* promoter, which is derepressed by the diauxic shift to growth on ethanol. The utilisation of medium supplements for xylanase production, rather than biomass formation, could thus be maximised. The culture pH was maintained at 5.0, unless otherwise specified.

Analytical methods

Cell density was determined by measuring absorbence at 620 nm after diluting samples with 9 g l^{-1} NaCl into the 0.05–0.2 linear detection range of the spectrophotometer. Absorbency measurements were calibrated to dry weight measurements made in parallel. Glucose, ethanol and succinate concentrations were determined by column liquid chromatography (CLC) in a Gilson CLC system (Middletown, Wis.). Compounds were separated on an HPX87-H column (Bio-Rad, Richmond, Calif.) at 45°C, with 5 mM H_2SO_4 at 0.6 ml min^{-1} as mobile phase, and detected with a Shimadzu RID6A refractive index detector (Kyoto, Japan). Samples were assayed for xylanase activity according to Bailey et al. (1992). The substrate [1% birchwood xylan (Sigma, St. Louis, Mo.) suspended in 50 mM citrate buffer, pH 6.0] and enzyme (diluted with 50 mM citrate buffer, pH 6.0) mixtures were incubated for 5 min at 60°C, and the reducing sugar determined (Miller et al. 1960); 1 unit (U) enzyme activity corresponded to 1 μmol reducing sugar released per minute. All enzyme activities were converted to protein amounts (milligrams) by using the specific activity of 1.812 (U $\mu g_{pure\ xylanase}^{-1}$), determined with purified protein (Görgens et al. 2001). For the quantification of intracellular xylanase activity, a sample of the fermentation broth was collected on ice, washed twice with 9 g l^{-1} NaCl and the cellular protein extracted with Y-PER (Yeast Protein Extraction Reagent; Pierce, Rockford, Ill.). Extracellular free amino acid concentrations were determined by ion exchange chromatography with postcolumn derivatisation (Biochemistry and Nutrition, DTU, Lyngby, Denmark; Barkholt and Jensen 1989). The ammonium ion concentration was determined using the Boehringer Mannheim Ammonia test kit (Cat. Nr 1112732), adapted for use with a Cobas Mira autoanalyser.

Results

The supplementation of defined medium with amino acids or succinate for the improvement of extracellular and intracellular heterologous xylanase production and biomass formation by a prototrophic, recombinant *S. cerevisiae* strain was investigated. Components were screened in shake-flask cultivation, prior to quantification in batch and continuous culture.

Screening of amino acids in shake-flask cultivation

Individual amino acids were added to shake-flask cultures at a concentration of approximately 4 mM, and at a time point near to glucose depletion, thus maximising their availability during the xylanase production (growth on ethanol) phase of the recombinant strain (Görgens et al. 2001). The total effect of individual amino acids on the extracellular xylanase production was expressed as relative xylanase productivity, which was calculated as the sum of the normalised values of the maximum volumetric xylan-

Table 1 Effect of individual amino acids on the extracellular xylanase productivity by a prototrophic transformed *Saccharomyces cerevisiae* strain at pH 3.0 in shake flask culture

Amino acid[a]	Cell density integral[b]	Volumetric activity[c]	Specific activity[d]	Specific activity integral[b]	Relative productivity[e]
Alanine	29	54	64	60	207
Arginine	100	100	100	100	400
Asparagine	45	85	88	90	308
Aspartate	3	−21	8	7	−4
Cysteine	−74	−94	−80	−34	−282
Glutamate	40	54	71	82	246
Glutamine	64	96	98	91	349
Glycine	25	30	44	25	124
Histidine	−1	−36	−3	−6	−46
Isoleucine	−132	−105	−99	−71	−407
Leucine	−28	−68	−49	−26	−171
Lysine	−4	−47	−19	−17	−86
Methionine	−110	−103	−83	−41	−338
Phenylalanine	−69	−85	−56	−34	−244
Proline	21	−8	27	15	55
Serine	14	−11	16	12	31
Threonine	−50	−77	−54	−32	−212
Tryptophan	−116	−104	−85	−45	−350
Tyrosine	−22	−64	−37	−23	−146
Valine	−6	−53	−27	−18	−104
Control	0	0	0	0	0

[a]Amino acids were added to a final concentration of approximately 4 mM
[b]Time-wise integrals of the cell density and the specific xylanase activity for the course of the cultivation (normalised values)
[c]Normalised values of the maximum volumetric xylanase activity for each culture (U ml^{-1})
[d]Normalised values of the maximum specific xylanase activity for each culture (U g_{cells}^{-1})
[e]The sum of the normalised values of the four components of the relative productivity. Maximum value corresponds to a 2.5-fold increase in the specific xylanase activity (U $mg_{biomass}^{-1}$)

ase activity (U ml^{-1}), the maximum specific xylanase activity (U g_{cells}^{-1}), and the time-wise integrals of the specific xylanase activity and the cell density (Tables 1, 2). First, the effect of amino acids on proteolytic degradation of extracellular xylanase was determined by cultivating the recombinant yeast in shake-flasks at pH 3.0, where increased extracellular protease activity has been observed (Ogrydziak 1993; Jones 1991). Subsequently, the effect of amino acid supplementation was determined at pH 5.0, where extracellular protease activity was reduced.

Supplementation of the defined medium with individual amino acids during cultivation at pH 3.0 dramatically improved extracellular xylanase levels (Fig. 1, Table 1). Three typical responses to amino acid supplementation

Table 2 Effect of individual amino acids on the extracellular xylanase productivity a prototrophic transformed *S. cerevisiae* strain at pH 5.0 in shake flask culture

Amino acid[a]	Cell density integral[b]	Volumetric activity[c]	Specific activity[d]	Specific activity integral[b]	Relative productivity[e]
Alanine	54	44	−4	4	98
Arginine	46	83	10	27	165
Asparagine	42	67	39	45	193
Aspartate	66	100	23	36	226
Cysteine	−477	−213	−12	−213	−916
Glutamate	34	−4	7	0	37
Glutamine	100	26	5	2	133
Glycine	11	81	28	50	170
Histidine	−282	53	69	84	−76
Isoleucine	−430	−261	−59	−292	−1042
Leucine	−83	18	17	11	−37
Lysine	15	73	19	22	128
Methionine	−308	33	80	25	−170
Phenylalanine	−75	53	29	−8	0
Proline	92	60	2	−10	145
Serine	−47	27	38	76	94
Threonine	−389	61	100	94	−135
Tryptophan	−172	−54	4	−1	−223
Tyrosine	−116	−36	26	21	−104
Valine	−276	−9	51	100	−134
Control	0	0	0	0	0

[a]Amino acids were added to a final concentration of approximately 4 mM
[b]Time-wise integrals of the cell density and the specific xylanase activity for the course of the cultivation (normalised values)
[c]Normalised values of the maximum volumetric xylanase activity for each culture (U ml^{-1})
[d]Normalised values of the maximum specific xylanase activity for each culture (U g_{cells}^{-1})
[e]The sum of the normalised values of the four components of the relative productivity

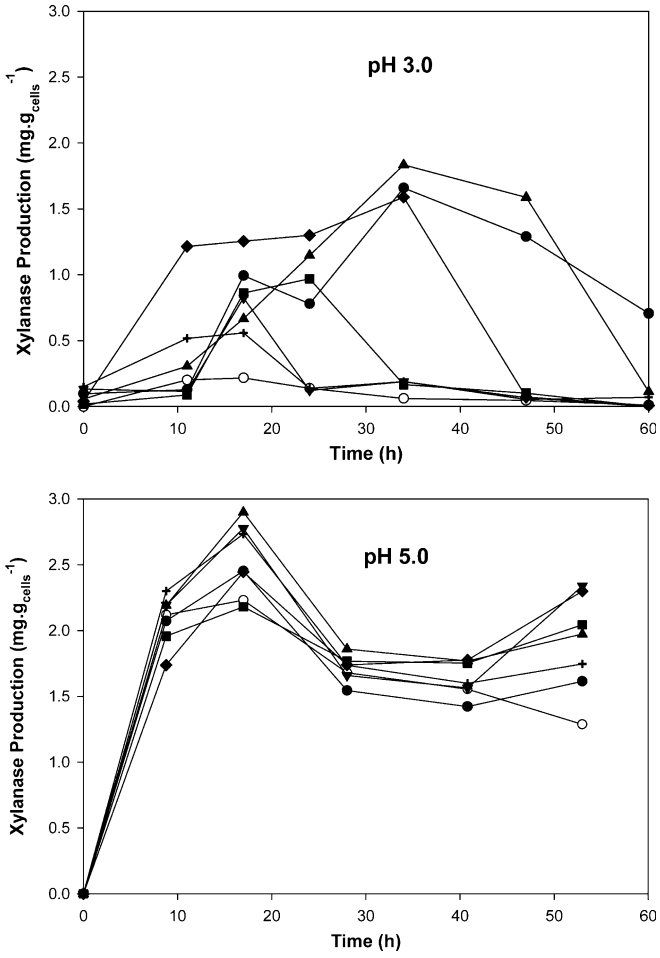

Fig. 1 Effect of individual amino acids on xylanase production by a prototrophic transformed *Saccharomyces cerevisiae* strain in shake-flask culture during growth on ethanol at pH 3.0 and 5.0. ○ Control without amino acid addition, ● glutamine, ▲ asparagine, ◆ arginine, ■ alanine, + glycine, ▼ glutamate (pH 3.0) or aspartate (pH 5.0). The pH of shake-flask cultures was controlled by either buffering defined medium to pH 5.0, using 50 mM citrate, or allowing the pH to decrease to approximately 3.0 during growth on glucose in unbuffered defined medium, due to ammonium utilisation (Greasham and Herber 1997)

were observed. Firstly, the amino acids Arg, Ala, Asn, Glu, Gln and Gly apparently retarded extracellular proteolysis of the xylanase protein, compared to the control, under the chosen conditions (Jones 1991). The increase in extracellular activity of proteases at low pH (Ogrydziak 1993; Jones 1991), present in the medium due to either secretion or cell lysis, resulted in degradation of the extracellular xylanase (Fig. 1a). In the control experiment, extracellular xylanase activity peaked after 18 h of cultivation, followed by loss of activity up to 30 h when levels were no longer measurable (Fig. 1a). Arg, Ala, Asn, Glu, Gln and Gly retarded the extracellular proteolysis of xylanase, resulting in higher maximum production levels and measurable xylanase activity up to 60 h of cultivation (Fig. 1a). Other responses involved the inhibition of either biomass (Ile, Trp, Met, Cys, Phe, Thr, Leu and Tyr) or xylanase production (Cys, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr and Val) by the prototrophic strain (Table 1).

Amino acid supplementation had a less dramatic effect on extracellular xylanase levels during cultivation at pH 5.0 (Fig. 1b), probably because the extracellular protease activity was reduced (Fig. 1b; Ogrydziak 1993; Jones 1991). The amino acids Ala, Arg, Asn, Asp, Gln, Gly and Lys improved the actual xylanase production by the transformed strain without inhibiting cell growth (Table 2). Biomass formation was inhibited by Cys, His, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr and Val during growth at pH 5.0, whereas significantly fewer amino acids (Cys, Ile, Trp, Tyr and Val) inhibited xylanase production (Table 2). The amino acids Ala, Arg, Asn, Glu, Gln and Gly reduced extracellular proteolysis (pH 3.0) and increased actual xylanase production (pH 5.0), and were selected for further testing under fermentation conditions in defined medium.

Amino acid quantification in batch fermentation

Batch cultures were supplemented with either arginine (40 mM total amino nitrogen), a four amino acid mixture (Arg, Asn, Ala, Gly; 20 mM total amino nitrogen) or a six amino acid mixture (Arg, Asn, Gly, Ala, Gln, Glu; 20 mM total amino nitrogen) during batch growth on ethanol (Fig. 2a–c; Table 3). Biomass formation, extracellular xylanase production (Fig. 2a, b) and ammonium consumption (not shown) increased due to supplementation with the four or six amino acid mixtures. These levels of biomass and xylanase production were comparable to those obtained with complex medium (Fig. 2a, b). The intracellular xylanase levels were improved as a result of amino acid supplementation (Table 4). The growth rate and rate of carbon (ethanol) consumption by the prototrophic transformant increased due to supplementation with the four or six amino acid mixture (Table 3; Fig. 2c).

Succinate addition to batch fermentation

The defined medium was supplemented with succinate after the shift from growth on glucose to growth on ethanol (Fig. 2d–f; Table 3), most of which was consumed within the first 32 h thereafter (Fig. 2f). Succinate consumption improved biomass formation and extracellular xylanase production (Fig. 2d, e). The biomass yield, maximum specific growth rate and intracellular xylanase levels of the recombinant strain increased (Tables 3, 4). The rate of ethanol consumption was not significantly influenced by the uptake of succinate (data not shown).

Quantification in continuous culture

In glucose-limited chemostat culture, the *ADH2* promoter regulating heterologous xylanase production by recombinant *S. cerevisiae* is derepressed during fully respiratory growth at low dilution rates (Du Preez et al. 2001). The effect of amino acid addition to continuous culture could therefore be determined by supplementing the defined feed

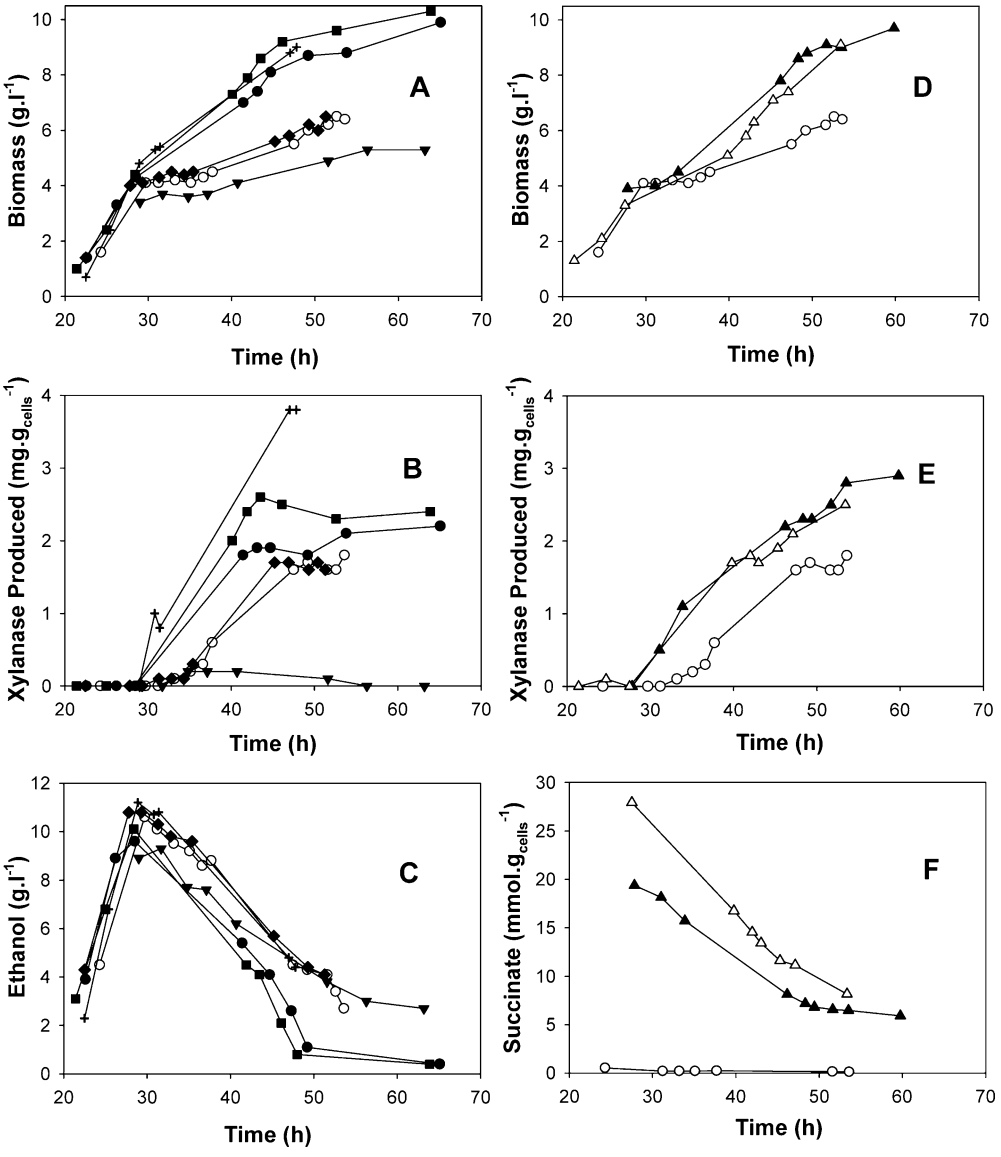

Fig. 2 Defined medium supplemented with either amino acid mixtures (**a**–**c**) at different total free amino nitrogen concentrations, or succinate (**d**–**f**), during growth on ethanol in batch culture. **a**, **d** Biomass; **b**, **e** xylanase; **c** ethanol production. **f** Succinate consumption during growth on ethanol in batch cultivation. ○ (pH 5.0), ▼ (pH 3.0) Controls without amino acid or succinate addition, + complex (YPD) medium control. Addition of Arg, Asn, Ala, Gly mixture (20 mM; ●), arginine (40 mM; ◆), Arg, Asn, Gly, Ala, Gln, Glu mixture (20 mM; ■), or succinate [9 g l^{-1} (▲), 11 g l^{-1} (△)]. Amino acid concentrations indicate the total free amino nitrogen content of the medium after supplementation

Table 3 Product formation and substrate consumption by the prototrophic *S. cerevisiae* [*pDLG6*] strain during batch growth on ethanol with amino acid or succinate supplementation

Supplementation[b]	Maximum specific growth rate [h^{-1}]	Co-substrates[a]				Products[a]	
		Glycerol	Acetate	Succinate	Total amino acids[c]	Biomass	CO_2
No amino acids, pH 5.0	0.025	−0.18	−0.07	0.0	0.00	0.50	0.75
No amino acids, pH 3.0	0.019	−0.24	–	0.0	0.00	0.49	0.75
Arginine (40 mM)	0.020	−0.20	−0.10	0.0	−0.13	0.59	0.84
Arg, Asn, Ala, Gly (20 mM)	0.038	−0.04	−0.05	0.0	−0.09	0.62	0.66
Arg, Asn, Gly, Ala, Gln, Glu (20 mM)	0.043	−0.04	−0.18	0.0	−0.13	0.71	0.64
Low succinate	0.045	−0.12	−0.11	−0.14	0.0	0.67	0.69
High succinate	0.043	−0.25	−0.05	−0.14	0.0	0.73	0.70
Complex medium (YPD)	0.049	−0.04	−0.45	0.0	−0.40	0.81	1.09

[a]Product- and substrate yields (cmol $cmol_{ethanol\ consumed}^{-1}$) were calculated for the duration of growth on ethanol. Yields were corrected for ethanol evaporation, which was calculated from the degree of reduction balance
[b]Concentrations indicate the total free amino nitrogen content of the medium after supplementation
[c]Total co-consumption of amino acids ($cmol_{amino\ acid\ consumed}$ $cmol_{ethanol\ consumed}^{-1}$)

Table 4 Specific intracellular xylanase activity 20 h after supplementation

Supplementation	Specific intracellular xylanase activity ($mg_{active\ xylanase}\ g_{cell\ protein}^{-1}$)
No amino acids, pH 5.0	2.49
Arginine (40 mM)	2.46
Arg, Asn, Ala, Gly (20 mM)	3.49
Arg, Asn, Gly, Ala, Gln, Glu (20 mM)	3.80
Low succinate	4.91
High succinate	2.93

medium to steady-state, glucose-limited cultures (Table 5). Casamino acids and SD-optimised mixtures of pure amino acids have previously enhanced heterologous protein production by *S. cerevisiae* (Wittrup and Benig 1994). The supply of an excess of the auxotrophic 7 amino acid mixture, containing His, Leu, Trp, Asp, Glu, Ser and Gly, has improved heterologous xylanase production by an auxotrophic strain recombinant yeast (Görgens et al. 2004).

In the present study, the addition of the four amino acid mixture improved extracellular xylanase production, despite the low level of supplementation, similar to batch cultivation (Table 5). Supplementation with the six amino acid mixture improved both biomass and xylanase production during growth on glucose. In the six amino acid mixture, all of the amino acids were utilised effectively by the prototrophic strain (Table 5). However, xylanase production by the prototrophic strain was inhibited by supplementation of the feed medium with the casamino acids and SD-optimised mixtures, despite the positive effect of casamino acids on biomass formation (Table 5). The auxotrophic 7 aa mixture (see above) inhibited both xylanase and biomass production by the prototrophic transformant in glucose-limited chemostat culture, as was observed elsewhere (Görgens et al. 2004).

Discussion

The supplementation of a chemically defined medium with amino acids or succinate for the improvement of heterologous xylanase production by a prototrophic *S. cerevisiae* strain was investigated. Amino acids were first screened in shake-flask culture, prior to the testing of combinations in batch and continuous cultivation. Supplementation of the defined medium with suitable amino acid mixtures or succinate significantly improved xylanase production by recombinant *S. cerevisiae*.

Amino acid supplementation

The screening of all 20 amino acids indicated that heterologous xylanase production could be improved by supplementation with Ala, Arg, Asn, Glu, Gln and Gly. Among these amino acids, Gln and Asn are classified as

Table 5 Yields of metabolic products ($cmol_{product}\ cmol_{glucose\ consumed}^{-1}$) and xylanase ($mg_{xylanase}\ cmol_{glucose\ consumed}^{-1}$) during amino acid supplementation of the feed to glucose-limited chemostat cultures of the prototrophic *S. cerevisiae* [*pDLG6*] strain

Amino acid supplementation[a]	Co-substrates		Products			
	Total amino-N (mM)[a]	Amino acid uptake[b]	Biomass	CO_2	Ethanol evaporation	Xylanase[g]
None	0	0	0.55	0.35	0.055	31.86
Casamino acids[c]	20	–	0.74	0.39	0.054	12.23
SD-optimised[c]	20	–	0.59	0.42	0.054	3.81
Auxotrophic 7 aa mixture[d]	20	–	0.43	0.59	0.035	0.23
Auxotrophic 7 aa mixture[d,e]	20	–	0.40	0.55	0.030	3.42
Arginine	8	–	0.58	0.38	0.028	32.93
Arginine (59.1)[f]	20	0.051	0.63	0.37	0.025	31.97
Arginine	40	–	0.63	0.46	0.047	22.14
Arg (48.1), Asn (99.8)[f]	20	0.069	0.63	0.48	0.00	24.85
Arg (59.1), Gln (97.9)[f]	20	0.081	0.60	0.38	0.054	29.31
Arg, Asn	40	–	0.66	0.51	0	38.07
Arg, Asn, Ala, Gly	2.5	–	0.56	0.34	0.064	37.22
Arg (99.4), Asn (99.8), Gly (95.1), Ala (82.7), Gln (99.1), Glu (99.1)[f]	20	0.132	0.68	0.41	0.016	49.29

[a]Different amino acid mixtures added to a feed medium containing 10 g l^{-1} glucose as carbon source
[b]Amino acid consumption at steady-state
[c]Pure amino acids added according to (Wittrup and Benig 1994)
[d]Görgens et al. (2004)
[e]Feed medium contained only 5 g l^{-1} glucose
[f]Percentage of the amino acid supply in feed utilised by the yeast indicated in brackets
[g]$mg_{xylanase}\ cmol_{glucose\ consumed}^{-1}$

preferred nitrogen sources because they are consumed readily from defined medium, despite the presence of ammonium (Slaughter et al. 1990; Jiranek et al. 1995; Ter Schure et al. 2000; Wiame et al. 1985; Cooper 1982). Arg and Glu have high molar contents of nitrogen, allowing their preferential utilisation from mixtures (Jiranek et al. 1995; Herraiz and Ough 1993), leading to improved heterologous xylanase and biomass production. The beneficial effect of the selected amino acids was strongly related to the inhibition of extracellular proteases (Kang et al. 2000; Coppella and Dhurjati 1989; Boze et al. 2001; Werten et al. 1999; Goodrick et al. 2001; Sreekrishna et al. 1997), especially during screening at pH 3.0 (Fig. 1a; Ogrydziak 1993; Jones 1991). The selected amino acids Arg, Gly, Ala, Asn and Gln are preferred for storage in the vacuole (Messenguy et al. 1980; Wiame et al. 1985; Grenson 1992; Horák 1997).

Supplementation with succinate

The availability of the TCA cycle intermediate succinate, limited biomass formation and protein production during growth on ethanol. Most of the succinate supplemented to the defined medium was consumed during batch growth on ethanol (Fig. 2f). Improved xylanase and biomass production were thus related to the actual consumption of succinate, and not to its buffering capacity (Adams et al. 1989), which dominated a previous investigation into its use as a medium supplement (Cha et al. 1998). The addition of succinate to cultures without pH control (Cha et al. 1998) will halt the characteristic reduction in pH during the course of the cultivation. This will minimise the extracellular proteolysis of the gene product at low pH (Ogrydziak 1993; Jones 1991) and result in apparently higher production levels. In the present investigation, the influence of the succinate buffering capacity on xylanase production was avoided by careful maintenance of the pH of control cultures. The positive effect of succinate consumption contradicts previous claims that extracellular TCA intermediates cannot support *S. cerevisiae* growth (Kaclikova et al. 1992).

The present fermentation results indicated that the availability of exogenous amino-nitrogen had a positive effect on protein production and biomass formation during growth on ethanol, as was previously observed (Chen et al. 1993; Gu et al. 1991; Albers et al. 1996). The positive effect of amino acid supplementation on xylanase production was observed only when a balanced mixture of individual amino acids was supplemented. The production of heterologous proteins may therefore be partially limited by the availability of metabolic precursors in carbon- or nitrogen metabolism during growth on ethanol or glucose, requiring empirical medium optimisation for new production strains. Alternatively, the supply of exogenous amino acids may also have improved the supply of tRNA loaded with particular amino acids, and thereby improve xylanase production. During growth on glucose in continuous culture, the presence of the amino acids, His, Ile, Leu, Met, Phe, Ser, Thr, Tyr and Val in the auxotrophic 7 amino acid, casamino acid and SD-optimised mixtures, inhibited xylanase production (Table 5). Some of the supplemented amino acids had a strong inhibitory effect on either biomass formation or heterologous protein production, and supplementation of yeast cultures with Cys, His, Ile, Leu, Met, Phe, Ser, Thr, Trp, Tyr and Val should probably be avoided. The disparity between the reported stimulation of heterologous protein production by the latter two mixtures (Wittrup and Benig 1994) and the inhibition of xylanase production observed in the present study, emphasises the need for empirical optimisation of defined media for each yeast production system.

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