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Differentiation Between Amino Acids Used as Carbon and Energy Sources During Growth of *Geotrichum candidum* Geo17

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Summary

Geotrichum candidum Geo17 was cultivated on peptones as carbon and nitrogen source, and in the presence of lactate as the second carbon source. From the analysis of the initial and final culture medium after total hydrolysis, the yield of consumption was determined for each amino acid. Amino acids have been considered a convenient carbon source for biosynthesis, while the rest of the amino acids were assumed to be used only as a nitrogen source, with the corresponding carbon released as CO_2 resulting from energy supply. Carbon mass balances confirmed this assumption. A clear differentiation between the amino acids assimilated as carbon sources and those assimilated as energy sources was therefore highlighted.

Key words: amino acids, carbon and nitrogen sources, *Geotrichum candidum*, growth

Introduction

The yeast *Geotrichum candidum* is involved in the neutralization of the curd during the ripening of Camembert cheese (*1*). This step plays an important role in the development of texture (*2*) and the further growth of surface bacterial populations (*3*). Curd neutralization results from both lactic acid assimilation (*4*) and ammonia release due to the deamination of amino acids (*5*). Metabolization of carbon and nitrogen substrates appears therefore as a key-factor in the ripening process, especially that related to the assimilation of amino acids.

In order to investigate the nutritional mechanisms involved in the assimilation of carbon and nitrogen sources by *G. candidum*, submerged cultures of the fungi have been carried out (*6*). Lactate as a carbon source and peptones as a carbon and nitrogen source have been used to simulate the aqueous phase of Camembert cheese (*7*).

Peptones are a complex mixture of various peptides. The amino acids constitutive of these peptides may be assimilated not only as nitrogen sources (for the main part of them), but also as carbon sources, as previously shown (*8*). Only general information may be deduced from the total amount of peptones assimilated (*6*); identification of the amino acids assimilated during culture of *G. candidum* (analysis of the culture medium after total amino acid hydrolysis) may be more informative. The differentiation between the amino acids which are convenient carbon sources and those assimilated only as nitrogen sources was also examined in this paper.

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Materials and Methods

Microorganism and inocula

The strain *Geotrichum candidum* Geo17 (Rhodia Food, Dangé St Romain, France) was used. Inocula were prepared from freeze dried spores stored at 7 °C.

Inoculation of the culture medium was carried out by an aseptic addition of spore suspension, namely the material consisting of arthroconidia (corresponding to an initial density of $2–3 \cdot 10^8$/mL). Arthroconidia were left to rehydrate for approximately 1 h in the sterilised medium at room temperature before inoculation.

Media

For the culture in fermentor, the medium basis was 5 g/L of tryptic casein peptones and 5 g/L of pancreatic casein peptones (Biokar, Pantin, France). When needed, the medium was also supplemented with 10 g/L of L(+) sodium lactate prepared by neutralisation of L(+)lactic acid (Acros, Geel, Belgium).

For the shake culture, the medium basis was 10 g/L of the selected amino acid.

Regardless of the mode of culture (fermentor or shake culture), the following components were also added in the media used: inorganic phosphates (P_i): KH_2PO_4 3.40 g/L and NaH_2PO_4 3.45 g/L (*9*); mineral solution containing the following ion concentrations (mg/L): ethylene diamine tetraacetate (EDTA) 585, Mg 25, Fe 20, Ca 18, Zn 4.5, Mo 2 and Cu 1.3 *(9)*. The pH of the media was then adjusted to 4.6 with 6M HCl, which is the pH of the lactic curd at the beginning of ripening (*7*).

Culture conditions

Shake cultures were carried out in 500-mL Erlenmeyer flasks containing 50 mL of the medium. The cultures were incubated at 20 °C on an orbital shaker (Unimax 2010, Heidolph, Kelheim, Germany) with an agitation speed of 200 rpm. Inoculation was made by an aseptic addition of 1 mL of spore suspension in the same medium (corresponding to a turbidity of 1.2 at 600 nm; spectrophotometer Helios Gamma Thermo Spectronic, Cambridge, England). Once or twice a day, samples of broth were harvested for pH measurements. At the end of the culture, total biomass was determined by dry weight measurement after filtration of the whole of the remaining broth on glass microfibre filters GF/C (Whatman, Maidstone, England).

Batch fermentations were carried out in a 700-mL laboratory-made glass-blown fermentor (*10*). The fermentor was filled with 300 mL of culture medium (the fermentor with the equipment and the medium were sterilized at 121 °C for 20 min).

Culture temperature was maintained at 25 °C by circulation of thermostated water in a jacket. The batch fermentor was continuously aerated with a constant airflow of 2.00 L/h (6.66 litre of air/litre of medium/hour) and the broth was magnetically stirred at 850 rpm. The fermentor was equipped with a sterilizable combination pH glass electrode (Ingold, Paris, France). The system also contained an aseptic recirculation loop involving a laboratory-made turbidimeter allowing on-line measurement of turbidity at 650 nm (*10*). Turbidity was calibrated from dry weight measurement of biomass at the end of culture. Carbon dioxide in the off-gas was also monitored on-line by an IR detector Rubis 3000 (Cosma, Igny, France).

Analyses

Total amino acid compositions of initial and final culture media were determined after hydrolysis with 6 M HCl at 110 °C under high-vacuum during 24 h (except cysteine and methionine, which were oxidized with performic acid and tryptophane hydrolyzed with 5 M NaOH). After hydrolysis, samples were diluted in mono-sodium citrate buffer (0.2 M, pH=2.2) then amino acids were analyzed after the separation on an ion exchange column Amersham LKB α-plus (Orsay, France), followed by reaction with ninhydrin (135 °C, 2 min) and colorimetric detection at 570 nm (except proline, which was detected at 440 nm). All the data given in Table 1 were the mean values of two determinations.

Results and Discussion

Geotrichum candidum Geo17 shake cultures on a single amino acid as the sole carbon and nitrogen source show that 6 amino acids (Ala, Arg, Asp, Glu, Pro, Ser) were assimilated as a carbon source, in addition to a nitrogen source (Table 2). As previously shown for *P. roqueforti* (*11*), the final amount of mycelium produced by *G. candidum* Geo17 followed the same order as the percentage of carbon in the amino acid (Table 2). It should be noted that 4 amino acids (Gly, Leu, Lys, Val) led to a significant growth in the presence of lactate in the medium, signifying that they can only be assimilated as nitrogen sources but not as carbon sources (*8*).

Medium alkalinization, always observed during *G. candidum* Geo17 growth on a single amino acid, even in the absence of lactate in the medium (Fig. 1), has to be related to the ammonium released, since amino acids contain excess nitrogen in relation to their carbon content for fungi, so that ammonium is released during their metabolization as C and N sources and it can raise the pH *(12)*.

It can also be assumed that, at least at the end of growth, most of the amino acids were taken up in exchange for OH^-, to maintain cellular electroneutrality. Indeed, the initial culture pH=4.6 was near the isoelectric pH of most of the amino acids (between 5 and 6). Therefore, at the beginning of growth, amino acids were mostly positively charged. However, due to the ammonium production, a culture pH close to the isoelectric pH was rapidly achieved. Then, and during a non negligible part of growth, amino acids were negatively charged since the culture pH was above their isoelectric pH, and their assimilation also contributed to the medium alkalinization. This was especially the case for the acidic amino acids (glutamic and aspartic acids), since they were negatively charged throughout the culture (pI near 3 for both amino acids); as illustrated for aspartic acid, since a high alkalinization rate was recorded from the beginning of growth, as well as a high final pH (Fig. 1),

Table 1. Total amino acid assimilation during cultures of *G. candidum* Geo17 on peptones and peptone-lactate based medium

	Amino acids	γ (medium initial)	Peptone-lactate			Peptones		
			γ(final)	γ(consumption)	γ(carbon)[c]	γ(final)	γ(consumption)	γ(carbon)[c]
		g/L	g/L	g/L	g/L	g/L	g/L	g/L
	Asp	0.603	0.264	0.339	0.1223	0.265	0.338	0.1220
	Thr	0.348	0.099	0.249	0.1004	0.097	0.251	0.1012
	Ser	0.415	0.175	0.240	0.0823	0.159	0.256	0.0878
	Glu	1.829	0.602	1.227	0.5008	0.568	1.261	0.5147
	Pro	0.779	0.214	0.565	0.2948	0.165	0.614	0.3203
	Gly	0.171	0.056	0.115	0.0368	0.053	0.118	0.0378
	Ala	0.259	0.057	0.202	0.0817	0.053	0.206	0.0833
	Cys	0.032	0.034	–0.002	–0.0006	0.041	–0.009	–0.0027
	Val	0.542	0.115	0.427	0.2190	0.115	0.427	0.2190
	Met	0.197	0.029	0.168	0.0677	0.029	0.168	0.0677
	Ile	0.422	0.103	0.319	0.1753	0.099	0.323	0.1775
	Leu	0.760	0.072	0.688	0.3781	0.065	0.695	0.3820
	Tyr	0.177	0.046	0.131	0.0782	0.047	0.130	0.0776
	Phe	0.428	0.049	0.379	0.2481	0.043	0.385	0.2520
	Lys	0.647	0.062	0.585	0.2885	0.055	0.592	0.2919
	His	0.230	0.067	0.163	0.0757	0.064	0.166	0.0771
	Arg	0.324	0.038	0.286	0.1183	0.035	0.289	0.1196
	Total	8.160		6.080	2.8700		6.210	2.930
γ(carbon source amino acid)[a]	g/L				1.20			1.25
Consumption	%				41.80			42.70
γ(nitrogen source amino acid)[b]	g/L				1.67			1.68
Consumption	%				58.20			57.30

[a] The convenient carbon source amino acids are Ala, Arg, Asp, Glu, Pro and Ser for *G. candidum* Geo17 (Table 2)

[b] All the amino acids except the carbon (and nitrogen) source amino acids

[c] Carbon from amino acid consumed, namely amino acid consumed/carbon content of the amino acid

while final biomass concentration was not especially high (1.5 g/L; Table 2).

Table 2. Comparison of the final biomass concentrations and the carbon content of the amino acids used by *G. candidum* Geo17 as carbon and nitrogen sources

	G. candidum					
Amino acid	Pro	Arg	Ala	Glu	Asp	Ser
$w(C_s)^a$/%	52.1	41.4	40.4	40.8	36.1	34.3
$\gamma(x_f)^b$/(g/L)	3.8	2.5	2.2	1.7	1.5	1.5

[a]carbon content of the amino acid
[b]final biomass concentration

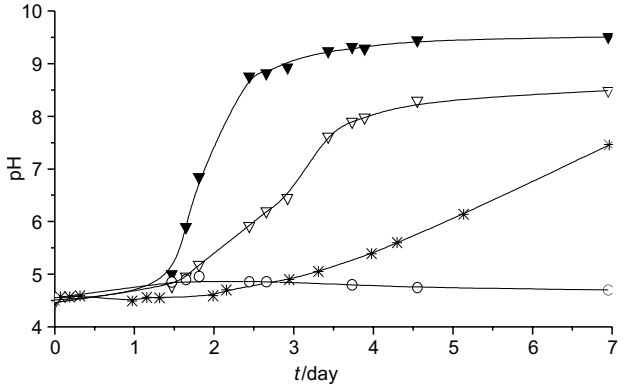

Fig. 1. pH time-courses during shake culture of *Geotrichum candidum* Geo17 growing on a single amino acid supplemented with inorganic phosphates and a mineral solution. (✳) Arg, (▼) Asp, (○) Cys, (▽) Pro

On the contrary, alkaline amino acids, like arginine (pI=10.76), were positively charged throughout the growth. It can be therefore assumed that, to maintain cellular electroneutrality, amino acids were taken up in exchange for H^+. This effect counterbalanced the alkalinization due to ammonium production, resulting in a low alkalinization rate and lower final pH (7.5; Fig. 1), while the final biomass concentration was high (2.5 g/L; Table 2).

During *G. candidum* Geo17 growth on peptones and lactate, the biomass concentration at the end of growth was considered (after approximately 60 h), *viz.* at the end of peptone consumption. Indeed, it had been previously shown that *G. candidum* Geo17 assimilated preferentially peptones over lactate as a carbon source, while lactate was mainly metabolized only as an energy source for cellular maintenance during stationary phase (*6*). Similar behaviour was therefore recorded regardless of the addition of lactate into the medium until the end of *G. candidum* Geo17 growth. The biomass concentration at the end of growth was also considered during the growth of *G. candidum* Geo17 on peptones, since the growth was immediately followed by a clear autolysis phase. Indeed, a lower amount of carbon was available due to the absence of lactate in the medium (*6*).

Biomass concentrations were 3.0 and 3.1 g/L at the end of growth on peptones and peptone–lactate, respectively. The carbon from biomass was therefore 1.15 and 1.19 g/L, since the carbon content of the cellular material of *G. candidum* Geo17 was 38.40 % (*6*).

For *G. candidum* Geo17 growing on peptones and peptone-lactate, the assimilation of each amino acid was determined from the difference between its initial and final concentration measured after total hydrolysis of the culture medium (Table 1). The amount of amino acids assimilated as carbon sources for *G. candidum* Geo17 was then determined by considering the convenient carbon source amino acids for this species (Ala, Arg, Asp, Glu, Pro, Ser), 1.25 and 1.20 g/L of carbon on peptones and peptone–lactate, respectively (Table 1). These results were in good agreement with the amount of carbon found in biomass (relative error = 4 and 0.5 %) for *G. candidum* Geo17 growing on peptones and peptone–lactate, confirming the above assumption.

The rest of the assimilated peptones, namely 1.7 g /L of carbon during *G. candidum* Geo17 growth on both media used (Table 1), was assumed to be used only as a nitrogen source, with the corresponding carbon released as CO_2 resulting from the energy supply. From this, in agreement with previous results (*13*) and regardless of the presence of lactate in the medium, similar amount of peptones was used for biosynthesis (42.2±0.4 %) during the growth of *G. candidum* Geo17 and the remainder (57.8±0.4 %) for the energy supply and released as CO_2.

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Razlike između pojedinih aminokiselina kao izvora ugljika ili energije tijekom rasta *Geotrichum candidum* Geo17

Sažetak

Geotrichum candidum Geo17 uzgajan je na peptonima kao izvoru ugljika i dušika uz laktat kao drugi izvor ugljika. Nakon potpune hidrolize podloge na početku i na kraju uzgoja, utvrđena je potrošnja svake pojedine aminokiseline. Pretpostavljeno je da su neke aminokiseline pogodan izvor ugljika za biosintezu, a ostale se koriste samo kao izvor dušika, pri čemu se ugljik otpušta u obliku CO_2 kao posljedica energetskih procesa. Ova je pretpostavka potvrđena na osnovi ustanovljene količine utrošenog ugljika. Stoga je jasna razlika između pojedinih aminokiselina kao izvora ugljika i onih koje se koriste kao izvor energije.