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SHORT COMMUNICATION

Sequential use of ammonium and leucine as nitrogen sources during growth of *Geotrichum candidum* on a glucose based medium

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Geotrichum candidum **growth on ammonium and leucine as nitrogen sources and glucose as a carbon source was examined. A clear preference of *G. candidum* for ammonium over leucine as a nitrogen source was shown. Indeed, ammonium was completely exhausted at the end of exponential growth after less than 35 hrs of culture; in contrast only 5% of leucine was concomitantly assimilated. Growth continued at slower rates on glucose and leucine as carbon and nitrogen sources respectively, and at the end of culture (185 hrs), leucine was completely exhausted.**

Geotrichum candidum is found in various habitats such as the soil, grass, silage, plants, fruits, feeding stuffs, insects, man and other mammals (Boutrou and Gueguen, 2005). It is also an important component of the microflora of soft cheeses such as Camembert and plays an important role in their maturation. Much attention has been paid to the biochemistry and the enzymatic activities of *Geotrichum candidum* (Boutrou and Gueguen, 2005), since it is involved in the development of the organoleptic characteristics of white soft cheeses (Boutrou et al. 2006) and their texturization through the neutralization of curd during ripening (Gripon, 2002). However, there is a lack of physiological studies, especially concerning the metabolization of amino acids, which are involved in curd neutralization through ammonium production resulting from amino acid deamination (Bonnarme et al. 2001). Indeed, amino acids contain excess nitrogen in relation to their carbon content for fungi, so ammonium is released during their metabolization as C and N sources (Deacon, 1997).

G. candidum can assimilate the majority of the amino acids as nitrogen sources and some of them as carbon sources (Amrane et al. 2003). Amino acid characterization based on their metabolic behaviour as carbon sources during *G. candidum* growth was then investigated (Amrane et al. 2003), leading to the characterization of three groups of amino acids, based on their carbon assimilation and dissimilation by *G. candidum*, in presence of a primary carbon source, glucose, as the limiting substrate (Aziza and Amrane, 2007). The first group (Cys, His, Phe, Thr and Trp) was only used as nitrogen sources by *G. candidum*, with glucose being the carbon and energy source. A glucose repression was shown for the rest of the amino acids, since only after glucose depletion, amino acids from the second group (Gly, Lys, Met, Val) were dissimilated for energy supply by oxidation into CO_2, while those from the third group (Ala, Arg, Asp, Glu, Leu, Pro and Ser) were assimilated as carbon sources, in addition to be used as nitrogen sources, leading to a diauxic growth.

From its discovery by Monod (1942), numerous works dealing with the sequential utilization of carbon substrates are available, described during growth on various substrates, like a mixture of sugars (Kremling et al. 2001), a mixture of hydrocarbons (Maachi et al. 2001) or sugar and alcohol (Jones and Kompala, 1999). Contrarily, only few works dealt with the sequential use of carbon sources

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involving amino acids, assimilated as both carbon and nitrogen sources, only after lactic acid exhaustion during *Brevibacterium linens* growth (Moreau et al. 1998), or glucose exhaustion during *P. camembertii* (Adour et al. 2005; Adour et al. 2006) or *G. candidum* growth (Amrane et al. 2003). A glucose repression (Ronne, 1995) has to be considered to account for the behaviour recorded during *G. candidum* growth on glucose and a single amino acid. It is thought to be an energy-saving response (Ronne, 1995), since glucose oxidation gives the best theoretical energy yield (38 molecules of ATP) (Deacon, 1997).

However, to our knowledge, examination of the behaviour recorded during growth on a mixture of nitrogen substrates has not been previously reported and was therefore examined in this work. The considered medium contained the most common nitrogen source, ammonium, and an amino acid from the third group, leucine, previously shown to be amongst the most efficient nitrogen sources for *G. candidum*, shown from the comparison of growth recorded on a wide range of amino acids, in presence of primary carbon sources, like glucose (Amrane et al. 2003) or lactate (Plihon et al. 1998). The comparison of an amino acid to a mineral and primary nitrogen source like ammonium may be helpful to complete the characterization of amino acids based on their metabolization and may improve the knowledge concerning the metabolization of amino acids, owing to the lack of related physiological studies.

MATERIALS AND METHODS

Microorganism

The commercial strain of *Geotrichum candidum*, Geo1*7* (Danisco, Dangé St Romain, France) was used. Freeze-dried spores were stored at +7ºC. In order to obtain reliable time lags, before inoculation spores were left to germinate approximately 1 hr at 25ºC in sterile culture medium. Spore viability was periodically controlled; the number of viable spores was determined by counting the Colony Forming Units having appeared after inoculation with successive decimal dilutions triplicates of pour plates of Yeast-Malt extract agar medium (Difco, Detroit, MI, USA) reconstituted at 38 g/L and incubated for 3 days at 20ºC.

Media

The medium used throughout this work contained: glucose, 8 g/L (Merck, Darmstadt, Germany); ammonium, 0.087 g/L, corresponding to 0.26 g/L NH_4Cl (Merck, Darmstadt, Germany); leucine, 0.63 g/L (Acros Organics, New Jersey, USA); inorganics phosphates (Pi): 25 mM of KH_2PO_4, and 25 mM of $NaH_2PO_4.H_2O$; and a solution of EDTA (Ethylene Diamine Tetra Acetate) (585 mg/L) chelated trace elements (mg/L): Mg, 25; Fe, 20; Ca, 18; Zn, 4.5; Mo, 2; Cu, 1.3.

The pH of the medium was adjusted to 4.6 with 6 M HCl, before it was sterilised at 121ºC for 20 min.

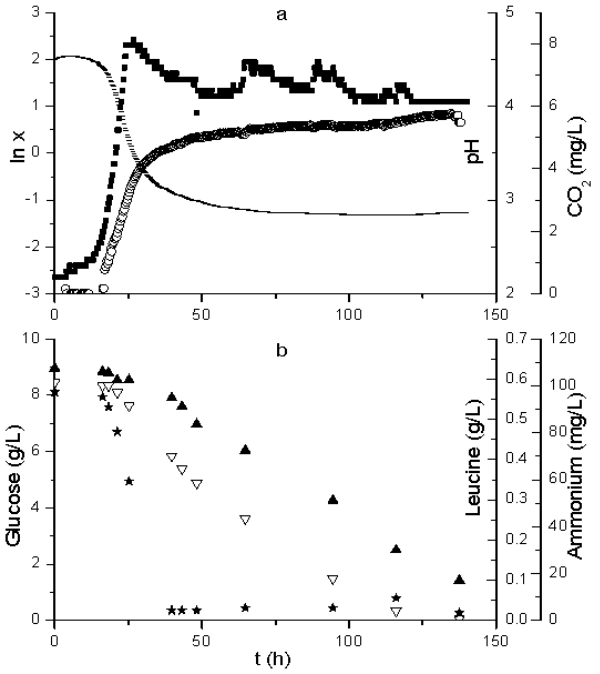

Figure 1. Culture data of *Geotrichum candidum* growing on ammonium, leucine and glucose based medium. (a) On-line monitored data: (○) biomass concentration; (-) pH; (■) CO_2 concentration; (b) Off-line data determined by periodical sampling: (▲) leucine, (★) ammonium and (▽) glucose concentrations.

Culture conditions

Batch fermentations were carried out in a 3 L laboratory-made glass-blown fermentor which was filled with 2 L of the culture medium. The fermentor equipments and the culture medium were sterilized at 121ºC for 20 min using a 90 L autoclave (Subtil-Crépieux, Chassieu, France).

During culture, the temperature was maintained at 25ºC by circulation of thermostated water in a jacket. The batch fermentor was continuously aerated with a constant airflow of 13.0 L/h (6.5 liter of air/liter of medium/h), and the broth was magnetically stirred at 850 RPM. The inoculation (10 mL) of the culture medium was carried out by an aseptic addition of spore suspension (corresponding to an initial density of 2-3 x 10^8 spores /mL). The product of the turbidity at 650 nm and the inoculum volume (A_{650}*V) was kept constant at a value of 13 for inocula; the number of spores was adjusted to achieve the considered value for the product A_{650}*V. Spores were left to rehydrate approximately 1 hr in the sterilized medium at room temperature before inoculation.

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 for on-

line measurement of biomass at λ = 650 nm (Aziza et al. 2004). Turbidity was calibrated from dry weight measurements of biomass at the end of culture. Biomass and broth turbidity were linearly correlated up to 5 g/L dry cellular weight, and the ratio turbidity on dry cellular weight was previously found to be 0.109, with a correlation coefficient of 0.98 (Amrane and Prigent, 1998). Carbon dioxide in the off-gas was also monitored on-line by an infrared detector Rubis 3000 (Cosma, Igny, France) after desiccation in a column of calcium chloride.

Analyses

After centrifugation of the samples (3000 g for 20 min), glucose, ammonium and leucine concentrations were determined in the supernatants. Glucose was enzymatically determined; it was first oxidized in pyruvate in the presence of glucose oxidase (GOD), and the produced hydrogen peroxide was measured after 80 min reaction with ABTS (AZINO-bis[3-EthylbenzThiazoline-6-Sulfonic acid]) in the presence of peroxidase (POD) (all from Sigma Diagnostics, St Quentin Fallavier, France). Leucine was determined on a 3% sulfonic acid filtrate using a Pharmacia LKB-Alpha Plus series 2 amino acid analyser (Pharmacia Biotech., Saclay, France) (Aziza et al. 2005). Ammonium was determined using a Dionex DX-500 high performance liquid chromatographic system (Dionex, F-78354 Jouy-en-Josas, France), with a cation exchange column (CS15 IonPac column, 250 x 4 mm) as described by Gaucheron et al. (1996). The observed standard deviations, deduced from triplicate measurements, were 0.3 g/L, 0.02 g/L and 4 mg/L for glucose, leucine and ammonium concentrations, respectively.

RESULTS AND DISCUSSION

Data displayed in Figure 1 are mean values deduced from duplicate runs. Logarithmic plot of growth time-course shows that after 15 hrs lag phase, in agreement with previous results recorded on a single amino acid and glucose (Aziza and Amrane, 2007), exponential growth was observed from 15 to 30 hrs of culture; then a late exponential phase was recorded until the end of culture (Figure 1a). It can be noticed that maximum specific growth rate of about 0.21 h^{-1} (Figure 1a) corresponded to that reported for an efficient amino acid, glutamate, and glucose (Aziza and Amrane, 2007). Noticeable production of CO_2 was recorded during lag phase of growth (Figure 1a), viz. during spore germination, in agreement with previous results (Aziza et al. 2004). The maximum for CO_2 production recorded after 27 hrs of growth was 8.1 mg/L (Figure 1a), can therefore be clearly related to the end of the exponential growth phase and then corresponded to the maximum for viable biomass (Adour et al. 2005), since CO_2 production resulted from the aerobic respiration of microorganisms. The decrease observed for CO_2 production during the late exponential growth phase remained relatively low, since after 120 hrs of culture, when glucose became limiting (Figure 1b), the instantaneous CO_2 concentration in the off-gases was 6.3 mg/L (Figure 1b).

The consumption of glucose, the carbon substrate, was linked to growth, since it became significant with the beginning of growth, and its exhaustion was obviously the cause of the cessation of growth, since it corresponded to the beginning of stationary state (Figure 1a) and a sharp decrease of the CO_2 emission (not shown).

It should be noted that medium composition was formulated to have similar amounts of nitrogen from ammonium and leucine, 0.068 and 0.067 g/L respectively. A clear preference of *G. candidum* for ammonium over leucine as a nitrogen source appears at the examination of Figure 1b. Indeed, ammonium was assimilated from the beginning of growth and was completely exhausted after less than 35 hrs of culture (Figure 1b). This lapse of time corresponded to the exponential growth phase and also included the maximum for viable biomass illustrated by the maximum for CO_2 emission (Figure 1a). During this lapse of time, a total amount of 0.03 g/L of leucine was taken up, namely only 5% of the available leucine (Figure 1b), which was completely exhausted at the end of culture (185 hrs - not shown). At the examination of Figure 1, ammonium consumption appeared clearly related to the medium acidification recorded. Medium acidification was previously reported during *G. candidum* growth on glucose and ammonia as C and N sources, respectively (Durand et al. 1973), and was attributed to a NH_4^+/H^+ exchange (Deacon, 1997). In addition to the contribution of ammonium to the acidification of culture medium, the consumption of leucine also contributed to the acidification, since culture pH were lower than the isoelectric pH of leucine, which was therefore positively charged throughout culture. Medium acidification was previously reported during amino acid assimilation, including leucine, as nitrogen sources, shown to be caused by an amino acid/H^+ exchange (Amrane et al. 2003; Adour et al. 2005). This behaviour was illustrated by the further acidification recorded after ammonium exhaustion from the medium (Figure 1a). The presence of ammonium was shown to prevent the synthesis of membrane-uptake proteins for other nitrogen sources (Deacon, 1997); this could account for the sequential use of nitrogen source shown in this work.

In conclusion, in presence of glucose as a carbon source, a clear preference of *G. Candidum* for ammonium over leucine was shown, illustrated by the sequential use of both nitrogen sources, even if leucine was previously shown to be an efficient nitrogen source for this species. Indeed, ammonium prevents the synthesis of membrane-uptake proteins for other nitrogen sources. To our knowledge, and contrarily to the sequential use of carbon substrates, that of nitrogen substrates was not previously reported.

To improve the knowledge concerning nitrogen assimilation by *G. candidum*, this study has to be followed

by similar work for other amino acids to complete the screening.

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