Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXIII, No. 11

LOW COST PRODUCTION OF PERDEUTERATED BIOMASS USING METHYLOTROPHIC YEASTS

Stéphane Haon[a], Sophie Augé[a], Marielle Tropis[a], Alain Milon[a]* and Nic D. Lindley[b]

[a] Laboratoire de Pharmacologie et de Toxicologie Fondamentales, CNRS, 118 rte de Narbonne, 31062 Toulouse, France.

[b] Département de Génie Biochimique et Alimentaire, URA CNRS N° 544, Lab. Ass. INRA, INSA, Complexe Scientifique de Rangueil, 31077 Toulouse, France.

SUMMARY

Three strains of methylotrophic yeasts, *Candida boidinii*, *Pichia angusta* (previously *Hansenula polymorpha*) and *Pichia pastoris*, were studied for their capacity to grow on methanol in deuterated media. Growth rates, determined relative to the extent of deuteration of water and/or methanol, showed that water deuteration was the major limiting factor. After adaptation to deuterium by progressive transfer through media of increasing deuteration, growth rates were diminished relative to those obtained on hydrogenated media of identical salts composition: the two *Pichia* species retained the highest growth rates (μ = 0.02 h^{-1}) in the fully deuterated medium. Perdeuterated biomass (16 g) was obtained in a 1 liter fed-batch fermentation and the extent of deuteration of isolated ergosterol has been shown to be 97.5% by mass spectrometric analysis .

Key words: deuterium, methylotrophic yeasts, fed-batch fermentation, perdeuterated lipids, ergosterol.

0362–4803/93/111053–11$10.50

Received 3 June, 1993
Revised 7 June, 1993

INTRODUCTION

Perdeuterated biomolecules are needed in many field of biophysics and biochemistry. In particular, NMR studies require partially deuterated proteins for extending the maximun range of sizes accessible to 2D NMR, specifically perdeuterated amino acids for simplifying the spectra, perdeuterated proteins for ligand-receptor interaction analysis and perdeuterated model membrane systems for transferred NOE determination of membrane bound conformations [1-8, for a review, see 3]. Simple molecules can be obtained efficiently by organic synthesis (e.g., pure perdeuterated phospholipids have been synthesized [9,10]), but for more complex molecules such as proteins, nucleotides, vitamins, coenzymes, sterols or natural lipid mixtures, the biotechnological approach is by far superior. Following the intensive work of Katz, Crespi and collaborators in the sixties who successfully demonstrated that most organisms can be adapted to growth in perdeuterated media [11-16; for a review see 14], several types of algae [17] and bacteria [4] have been used for production of perdeuterated metabolites and perdeuterated biomass coming from algal sources is commercially available (Cambridge Isotope Laboratories Inc., MA, USA). Algal sources are convenient because of their autotrophic metabolism which enables them to synthesise all their cell components from carbon dioxide and therefore the media require only deuterated water. The major disadvantage of algae is their relatively slow growth rates. While micro-organisms are capable of significantly increased growth rates, they usually require a growth medium containing more complex perdeuterated organic molecules (at least perdeuterated glucose) which are expensive in themselves. The perdeuterated carbon source often being obtained from an algal hydrolysate as was done by Katz and co-workers in the sixties.

In this work, eucaryotic biomass including sterols, was obtained at reasonably low cost, by growing methylotrophic yeasts [18-21] on a medium containing perdeuterated methanol as sole carbon source.

MATERIAL AND METHODS

Microorganisms and chemicals

Candida boidinii CBS 2428, *Pichia angusta* CBS 4732 and *Pichia pastoris* CBS 704 were used throughout this study. All chemical were obtained from usual commercial sources and used without further purification.

Culture media.

Two media were used throughout this study: standard growth medium (Medium M_1) and fed-batch medium (Medium M_2), both based on that formulated by Egli [21]. Medium M_1 contained per litre of distilled water: 4 g methanol, 1.5 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 1 ml of vitamin solution (SV_1: 5 mg biotine and 300 mg thiamine-HCl in 1 l of distilled water) and 2 ml of trace elements solution (TE_1: 50 g EDTA, 22 g $ZnSO_4.7H_2O$, 5.5 g $CaCl_2.6H_2O$, 6.6 g $MnSO_4.6H_2O$, 5.0 g $FeSO_4.6H_2O$, 1.1 g $(NH_4)_2Mo_2O_7$, 1.5 g $CuSO_4.5H_2O$ and 1.6 g $CoCl_2.6H_2O$ in 1 l of distilled water) Medium M_2 contained per litre of distilled water: 327 g methanol, 40.0 g $(NH_4)_2SO_4$ (both added continuously throughout the culture relative to the amount of biomass accumulated), 5.0 g KH_2PO_4, 1.0 g $MgSO_4$ $7H_2O$, 1 ml SV_2 solution (0.1 g biotine and 1 g thiamine-HCl in 1 l of distilled water) and 5 ml of TE_2 solution (50 g EDTA, 6 g $ZnSO_4.7H_2O$, 8 g $CaCl_2.6H_2O$, 0.6 g $MnSO_4.6H_2O$, 3.2 g $FeSO_4.6H_2O$, 0.06 g $(NH_4)_2Mo_2O_7$, 1.2 g $CuSO_4.5H_2O$ and 0.8 g $CoCl_2.6H_2O$ in 1 l of distilled water).

Adaptation of the cells to deuterium

Yeast cells were transfered from solid medium (M_1 medium containing agar at 15 g/l) to 5 ml of liquid M_1 medium. The cultures were incubated in plugged tubes at 30°C under reciprocal shaking (140 rpm) conditions. Once growth was well established a sample (0.5 ml) was aseptically transfered into identical medium of increased deuterated methanol and/or water content.

Hydrogenated and deuterated fed-batch cultures.

The cultivation were carried out at 30°C in a 1.5 l fermentator containing 1 l of M_2 medium with aeration (25 l of air per hour) and stirring (600-1300 rpm so as to maintain dissolved oxygen concentration above 50% saturation) at pH 6.0 maintained by automatic addition of NH_4OH). Methanol and $(NH_4)_2SO_4$ were added manually. Growth was followed turbidimetrically at 640 nm using a Hitachi spectrophotometer but was also calibrated to biomass dry weight by direct gravimetric measurements of washed culture samples filtered through nylon membrane filters (0.2 µm pore size) which were then dried to constant weight at 60°C under partial vacuum. The deuterated culture conditions were the same as those used for the hydrogenated culture except that methanol and water were 99.8% deuterated. The salts were also fully deuterated by repeated exchange with D_2O. Care was taken to remove water from the air supply by drying and then rehumidifying by bubbling through D_2O to limit exchange with H_2O.

Sterol analysis

The biomass was harvested by centrifugation at 12000 *g* for 15 min at 4°C. The cells were then broken by contact with liquid nitrogen and dried by azeotropic evaporation of water with toluene. The total lipids were extracted by refluxing in chloroform-methanol (2/1, v/v) for 5 hours. They were then subjected to saponification by 1 hour reflux in 25 ml of water and ethanol (1/4, v/v) saturated with KOH. The non polar lipids were then extracted with hexane (60 ml, three times). The sterols were purified by thin layer chromatography (TLC, eluent: dichloromethane), acetylated (pyridine-acetic anhydride 1/2; v/v, 12 hours) and the sterol acetates were purified again by TLC (eluent: dichloromethane). They were then analysed by gas chromatography on a SE-30 capillary column (gradient of temperature from 250°C to 300°C, 2°C/min) and GC-MS (HP 5989X, electron impact, 70 eV).

RESULTS AND DISCUSSION

Optimization of growth conditions on hydrogenated medium

The influence of the methanol concentration has been tested for the three strains between 0.1 and 1.25%. The best specific growth rate was obtained for all three strains at 0.5% methanol, pH 6, 30°C for *C. boidinii* and *P. pastoris*, and at 37°C for *P. angusta*.

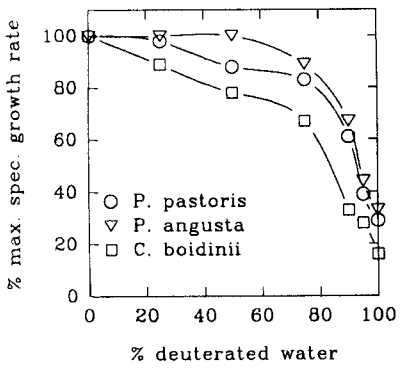
Adaptation to deuterium

The cell were adapted to increasing amounts of deuterium by sequential transfer through media containing 25%, 50%, 75%, 90%, 95% and >98% deuterium allowing growth to become stabilised in each medium (4 to 8 transfers) before passing to the next level. The specific growth rates associated with each medium were expressed in comparison with the specific growth rate in the M_1 medium with 100% hydrogenated components.

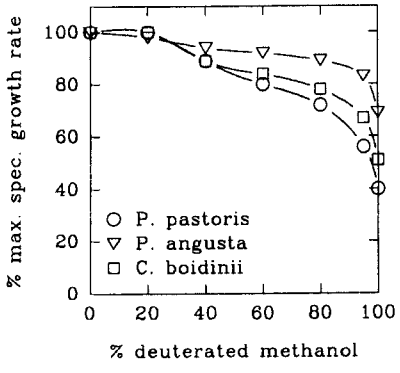
As for other organisms [13], the deuterated water was not a favourable medium for the growth of methylotrophic yeasts (Fig. 1A). For all three strains, an important fall of the specific growth rate ocurred between 75 and 100% deuterated water. The specific growth rate obtained at 100% was low in all cases, but *P. pastoris* and *P. angusta* seemed better adapted to this medium (33 and 29 %) than *C. boidinii* (15%).

The yeasts seemed to be significantly less sensitive to deuterated methanol than to deuterated water (Fig. 1B). In a medium containing 100% deuterated methanol, all strains showed specific growth rates superior to 40%: the best result was obtained with *P. angusta* (69 %).

In a medium containing both 98% deuterated water and 99.8% deuterated methanol, the yeasts reacted as for deuterated water (Fig. 1C). *C. boidinii* was very sensitive to this medium and the two other strains supported a specific growth rate equal to only 21% of that obtained in an otherwise identical hydrogenated medium.



A



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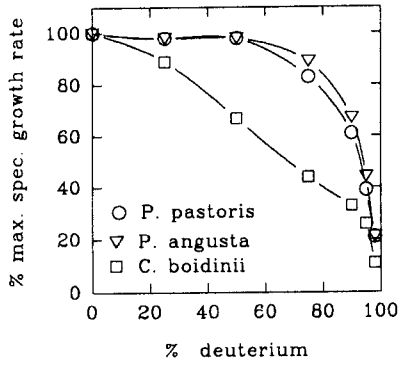

C

Figure 1: Specific growth rate of three methylotrophic strains in increasingly deuterated growth media. A) Deuterated water; B) Deuterated methanol; C) Both water and methanol deuterated.

Biomass production

P. angusta was selected for large scale biomass production because of its more rapid rates of growth and a unicellular rather than filamentous or pseudomycelial morphology. Medium M_2 was formulated in order to enable biomass synthesis to take place free of elemental limitations up to biomass concentrations exceding 100 g/l (composition based on that proposed in a theoretical study of growth requirement of methylotrophic yeasts [21]). Growth was allowed to develop free of external control in an exponential manner until the maximum rate of aeration and agitation compatible with the bioreactor's correct operation was reached. To avoid oxygen transfer becoming a limiting factor and hence growth under anoxic environments, the

rate at which methanol was added to the medium was maintained constant from this point onwards. Carbon limited growth was rapidly established in which further accumulation of biomass was of a linear nature and maintained growth conditions at an optimal level coherent with the limitations imposed by the bioreactor. It should be noted that in addition to the maintenance of a correct rate of oxygen transfer this control strategy also prevented the accumulation of metabolic heat (often a problem with high cell density cultures of methylotrophic yeasts).

Cultures in hydrogenated medium: A fixed rate of methanol addition (2.5 ml/h) was established after 32 hours of exponential growth at a biomass concentration of approximately 20 g/l and thereafter linear growth occurred with a final biomass concentration of 144 g/l being reached after 220 hours (Fig. 2A). During the final stages of growth, the biomass developement became limited by the metabolic maintenance requirements which accounted for the quasi-totality of the methanol added to the culture.

Culture in deuterated medium: As was observed in tube cultures during adaptation of the cells, the growth rate was significantly lower in deuterated medium (Fig. 2B). Because of this lower growth rate, the culture remained in exponential phase throughout the experiment since maximum rates of oxygen transfer were not reached. Under these fed-batch conditions in

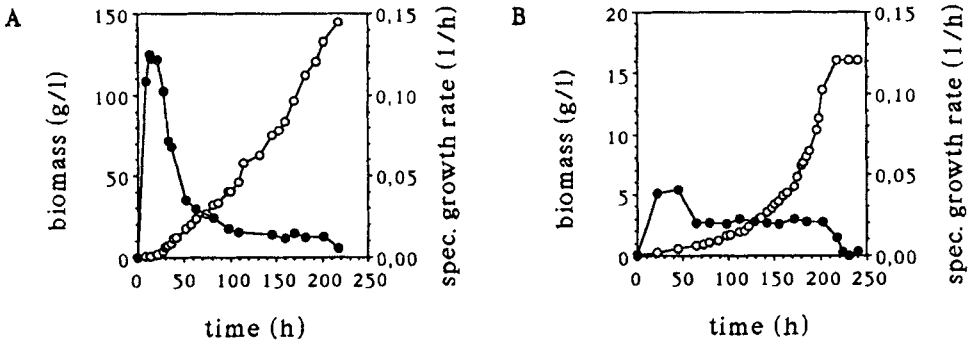

Figure 2: Specific growth rate and biomass obtained during fed-batch cultures of *P. angusta* in hydrogenated (A) or perdeuterated (>98%) medium (B). ○ biomass; ● specific growth rate

which methanol was always in excess, the culture developed 16 g/l of biomass after 250 hours, but thereafter no further growth occurred. The reasons for this premature halting of biomass synthesis have not yet been clearly identified and is currently under investigation.

Analysis of sterol components of the biomass.

P. angusta and other methylotrophic yeasts contain ergosterol as major sterol as is observed classically with *Sacchromyces cerevisiae* [22]. Ergosterol, acetylated during the purification process, was separated and identified by gas chromatography (identical relative retention time (RRT) compared with authentic cholesterol acetate) coupled to electron impact mass spectrometry [23]: MW = 438 (intensity 10%), fragments: 378 (100%), 363 (38%), 253 (64%), 211 (25%). Ergosterol (RRT = 1.07) represented 95% of the sterols and an additional minor sterol, which represented less than 5%, was identified as an 24Me-cholesta-tetraenol (MW = 436, RRT = 1.03). The same two sterols were found in similar proportions for the deuterated biomass samples from fed-batch cultures with RRTs equal to 1.00 and 1.04 for deuterated ergosterol (a slightly shorter RRT for the perdeuterated molecules is classical in gas chromatography, [24]). The mass spectrum of perdeuterated ergosterol is shown in Figure 3.

Due to the isotopic distribution, a complex spectrum was observed for each fragment: a statistical analysis of these patterns provides the average extent of deuteration of the molecule [25]. From the 16 g of dry biomass about 20 mg of ergosterol with a deuteration extent of 97.5% was obtained. This level of deuteration is fairly high considering that the culture lasted 250 hours and that some exchange of hydrogen may have occured with the environment. The composition of the polar lipids obtained from the same biomass (2.5 g) is currently being examined.

CONCLUSION

In this study the capacity of three strains of methylotrophic yeasts (*C. boidinii*, *P. angusta* and *P. pastoris*) to adapt to perdeuterated medium have

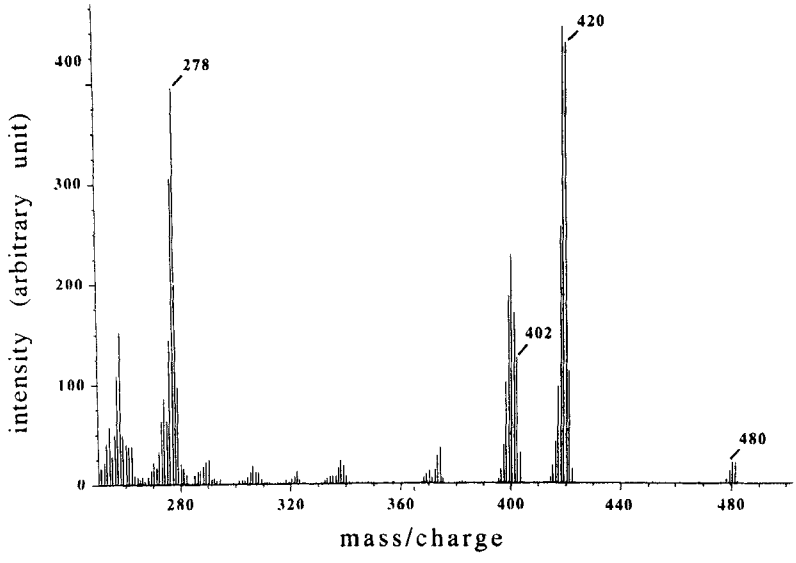

Figure 3: Mass spectrum of ergosterol acetate isolated from a fed-batch culture of *P. angusta* in perdeuterated medium. The isotopic distribution corresponds to an average deuteration extent of 97.5%.

been compared and the *Pichia* species shown to be best suited. In deuterated media their specific growth rates decreased rapidly with the extent of deuteration once the extent of deuteration excedes 75% and represents only 10-20% of that obtained in hydrogenated medium in a fully (more than 98%) deuterated medium. The possibility to easily produce a high level of *P. angusta* biomass (144 g/l) if the feeding strategy was adapted to the bioreactor's technical limitations was demonstrated for a hydrogenated medium and a fairly high level of biomass (16 g/l) was attained in a perdeuterated (97.5%) environment from which 20 mg of perdeuterated ergosterol have been obtained. This result can probably be further improved by analysing the possible limitations and long term toxicities specific to growth on deuterated medium. Rates of biomass production could certainly be significantly improved by decreasing the extent of deuteration to 95%, at which level growth rates would be increased twofold.

Methylotrophic yeasts have been demonstrated to be an excellent system for the production of perdeuterated biomass at relatively low cost. This was due in part to the economic advantages of deuterated methanol which is considerably cheaper than more complex organic feedstocks but also to the facility with which these organisms can be grown. In this study emphasis has been given to the production of sterols but of course the biomass produced also contains a wide range of desirable molecules whose extent of deuteration would logically be comparable with that of the ergosterol analysed. Moreover, these yeasts are good hosts for the expression of heterologous proteins and could provide a useful way of producing perdeuterated proteins in a eucaryotic expression system [26-29].

ACKNOWLEDGEMENTS: the Euriso-top society, Saclay, is gratefully acknowledged for its interest in this work and the gift of perdeuterated substrates. A. Milon is grateful to Dr Mermet Bouvier, Prof. G. Ourisson and Dr Y. Nakatani who made this work possible through initial advices.

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