ELSEVIER

PII: S0308-8146(96)00341-X

Food Chemistry, Vol. 60, No. 3, pp. 347–355, 1997

0308-8146/97 $17.00+0.00

Production of carotenoids by *Xanthophyllomyces dendrorhous* growing on enzymatic hydrolysates of prehydrolysed wood

J. C. Parajó, V. Santos, M. Vázquez & J. M. Cruz

Department of Chemical Engineering, University of Vigo (Campus Orense), Faculty of Sciences, Las Lagunas, 32004 Orense, Spain

(Received 6 September 1996; accepted 1 November 1996)

Milled wood samples were subjected to acid prehydrolysis under selected operational conditions to remove the hemicellulosic fraction, and the solid residues (directly or after oxidation with NaClO) were used as substrates for the enzymatic hydrolysis of cellulose. The glucose solutions obtained were supplemented with nutrients, sterilized and used for culturing the red yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*). Both cell growth and carotenoid production by the yeast were studied in culture media made from hydrolysates. The experimental results showed that the culture media made from hydrolysates are suitable for both proliferation and carotenoid production by the micro-organism studied. The distribution of individual carotenoids (including astaxanthin, canthaxantin, β-carotene and echinenone) in microbial pigments was also studied. © 1997 Elsevier Science Ltd

INTRODUCTION

Carotenoids are natural pigments with a variety of applications in food technology. Astaxanthin (AX, 3,3′ dihydroxy-ββ-carotene –4–4′ dione) is a carotenoid widely distributed in nature: for example, the characteristic coloration of salmon flesh, trout or shrimp is due to this compound. AX is also used as additive for poultry rations in order to improve the coloration of both flesh and egg yolks (Johnson *et al.*, 1980*a*,*b*; Johnson & An, 1991; Tangeras & Slinde, 1994).

Neither fish nor poultry are able to synthesize carotenoids, which must be provided in feed. Because of that, a large and growing market exists for AX. Other factors increasing the interest for AX production are its high market price (accounting for a significant part of salmon feed costs) and its efficient deposition in flesh (Torrissen *et al.*, 1989; Johnson & An, 1991).

AX can be produced by chemical or biotechnological procedures. The chemical way has been commercially developed by Hoffman-La Roche, whereas a variety of micro–organisms are able to synthesize AX. For example, algae (*Haematococcus pluvialis*), bacteria (such as *Agrobacterium auranticum, Mycobacterium lacticola* or *Brevibacterium* spp.) and yeast (such as *Xanthophyllomyces dendrorhous*, formerly *Phaffia rhodozyma*) are useful for this purpose (Andrewes *et al.*, 1976; Bubrick, 1991; Nelis & Leenheer, 1991; Tjahjono *et al.*, 1994; Yokoyama *et al.*, 1994). Among micro-organisms, yeasts are the most promising AX producers, notably for aquaculture applications (Fang & Cheng, 1993).

Phaffia rhodozyma can grow from a variety of carbon sources, including glucose, maltose, saccharose, cellobiose, xylose, arabinose and lactose (Johnson & An, 1991; Fang & Cheng, 1993). Additionally to standard sugar solutions, culture media made from plant or fruit juices (Okagbue & Lewis, 1984; Meyer & du Preez, 1994*a*), byproducts of corn wet milling (Hayman *et al.*, 1995) or acid hydrolysates of lignocellulosic materials (Martin *et al.*, 1992, 1993) have been used for making proliferation media. To our knowledge, enzymatic hydrolysates have not been tested as components of culture media for carotenoid production by yeast or bacteria.

Enzymatic hydrolysis of lignocellulose is a promising way to use this renewable, cheap and widespread resource. Wyman (1994) reported a comparative evaluation of saccharification technologies, and concluded that enzymatic hydrolysis is now beginning to appear economically advantageous. For this purpose, cellulases from the fungus *Trichoderma reesei* is the most widely employed enzymatic complex.

In the enzymatic hydrolysis of biomass, the reaction is carried out under mild experimental conditions, leading to easily fermentable sugar solutions. However, native lignocellulosic materials are composed of cellulose fibres

with crystalline structure that are embedded in a matrix composed by hemicellulose and lignin. Because of that, the chemical resistance and the lack of accessibility to the substrate limit both the yields and kinetics of saccharification. In order to improve these factors, the raw material can be pretreated. The pretreatment steps increase both operational costs and investment, but also improve the rate and yield of saccharification, enhancing the economic features of the process.

Among the pretreatments assayed, the dilute acid process appears to stand best in economic terms (Wyman, 1994). In a single step, this technology causes several alterations in raw materials, including cellulose decrystallinization, hemicellulose removal, dissolution of acid-soluble lignin, increase in pore volume and available surface area; all of these improve subsequent enzymatic hydrolysis. Under selected operational conditions, the cellulose is almost quantitatively retained in the solid phase during the dilute acid treatment (Parajó *et al.*, 1995*a*).

On the other hand, the hemicellulosic sugars generated by these treatments can be used as carbon sources for the fermentative production of food-related products. For example, when the raw material is a hardwood, the xylose-rich solutions obtained from hemicelluloses can be used for producing xylitol (Heikkila *et al.*, 1992; Parajó *et al.*, 1996), whereas the sugar solutions generated from softwoods (a mixture of xylose, arabinose, glucose, mannose and galactose) can be fermented to Single-Cell Protein by *Scytalidium acidophilum* (Ivarson & Morita, 1982). Figure 1 shows the principle of a possible process based on the utilization of a softwood as a source of hemicellulosic sugars and glucose.

According to the ideas above, the solid residue from dilute acid hydrolysis (prehydrolysis) shows an enhanced susceptibility to enzymatic hydrolysis in comparison with native lignocellulose. However, depending on the raw material, the effects caused could be insufficient to produce a highly reactive substrate for enzymatic conversion. Some resistant substrates show poor accessibility even after the acid prehydrolysis, and a subsequent treatment with oxidizing agents is necessary. The coupling of sequential steps with acid and NaClO results in a processing scheme that compares very well in efficiency with other reported chemical pretreatments (David *et al.*, 1988).

This work reports on a process including the following stages: chemical processing of wood, enzymatic hydrolysis of the solid residue from treatments and carotenoid production by *Xanthophyllomyces dendrorhous* in culture media made from the above hydrolysates. Pine wood was treated with sulphuric acid under selected operational conditions (Parajó *et al.*, 1995*a*) in order to remove the hemicellulosic fraction, and the solid residues obtained were evaluated as substrates for enzymatic hydrolysis. Additional experiments were carried out in order to study the improvements obtained in the saccharification step when an oxidizing treatment was performed sequentially to the treatment with sulphuric acid. The enzymatic hydrolysates were used for proliferating cells of *Xanthophyllomyces dendrorhous*, and both the fermentation dynamics and the carotenoid profile were determined. For comparative purposes, similar experiments were carried out using commercial glucose solutions instead of wood hydrolysates.

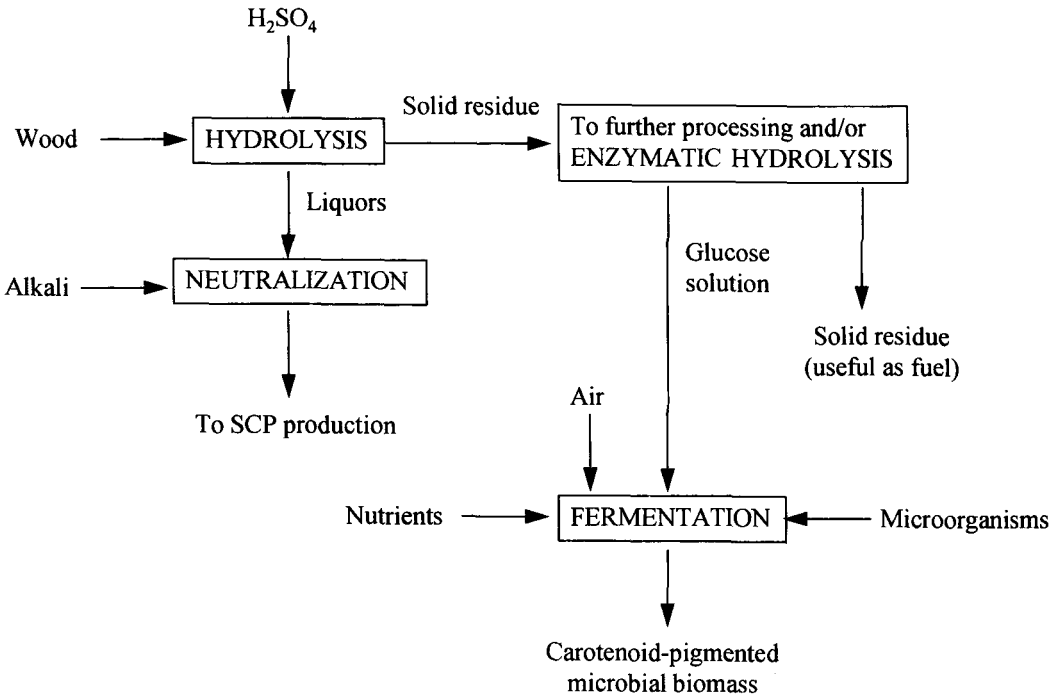

Fig. 1. Principle of a possible process based on the utilization of both hemicellulosic sugars and cellulose for obtaining food-related products.

MATERIALS AND METHODS

Raw material and chemical processing

Pinus pinaster wood chips were collected in a local pulp mill, milled to pass a 1 mm screen, homogenized in a single lot and stored. Samples from the above lot were treated with dilute sulphuric acid solutions under operational conditions selected on the basis of a previous study (Parajó *et al.*, 1995*a*). In a first set of experiments, the solid residues from these treatments were used as substrates for enzymatic hydrolysis. In other assays, the solid residues were treated with NaClO solutions before the enzymatic hydrolysis stage (see below). The composition of cellulosic substrates was established by means of a normalized, quantitative acid hydrolysis with 72% sulphuric acid (Browning, 1967). The content in lignin was determined gravimetrically, whereas cellulose and hemicelluloses were measured from the hydrolysates by HPLC as reported elsewhere (Parajó *et al.*, 1995*b*).

Enzymatic hydrolysis

The cellulosic substrates from the above treatments were hydrolyzed in media containing a mixture of cellulases from *Trichoderma reesei* (Celluclast) and cellobiase from *Aspergillus niger* (Novozym). The enzyme concentrates were kindly provided by Novo (Denmark). The activities of enzymes in the reaction media and the liquor/solid ratio were considered as operational variables, and all the enzymatic runs were performed during 48 h at 48.5°C and pH 4.85 (citrate buffer 0.05 N) using a liquor/solid ratio of 20 g/g. At given reaction times, samples were withdrawn from the reaction media and analyzed for glucose by HPLC (Parajó *et al.*, 1995*b*).

Organism and fermentation assays

Freeze-dried broths of *Xanthophyllomyces dendrorhous* ATCC 24228 were proliferated in a medium containing 10 g glucose/l, 3 g yeast extract/l, 3 g malt extract/l and 5 g peptone/l, and transferred to plates containing the same medium supplemented with 20 g agar/l. Sugar solutions (enzymatic hydrolysates or commercial glucose) were supplemented with nutrients as above, sterilized, inoculated with loopfuls of *Xanthophyllomyces dendrorhous* and incubated in orbital shakers. The experimental conditions for fermentation were agitation speed = 200 rpm, temperature = 21°C and initial pH = 6. At given fermentation times, samples were taken from the culture media and centrifuged. Glucose was determined in supernatants by the same method used for enzymatic hydrolysates, and the pellets were washed twice with sterile water and used to determine both biomass (as dry weight) and carotenoids using separate aliquots.

Carotenoid determination

Carotenoids were extracted from cells by disruption with DMSO (Sedmak *et al.*, 1990) and transferred to hexane (Calo *et al.*, 1995). After centrifugation, samples were analysed by HPLC using a diode array detector (Hewlett Packard 1050 Series). The analyses were carried out using a Merck LiChrosorb Si 60 column (oven temperature = 30°C) using gradient elution (flow rate = 1 ml/min; mobile phase: 100% hexane during 1 min; change up to 50% hexane and 50% ethyl acetate in 2 min, this last concentration remaining constant during additional 6 min). Carotenoids were identified by their retention times and by comparison of the vis-UV spectra with those of pure compounds. AX and echinenone standards were kindly provided by Hoffman-La Roche.

RESULTS AND DISCUSSION

Dilute acid hydrolysis (prehydrolysis)

The kinetics and product distribution of the acid prehydrolysis of pine wood have been previously studied (Parajó *et al.*, 1995*a*). Table 1 shows the raw material composition and the set of operational conditions selected on the basis of our previous experience, as well as other data on the residue yield and composition of phases. It can be noted that under the harsher conditions, the cellulose degradation is negligible whereas an almost complete hemicellulose removal is achieved, and that the hydrolysates contain up to 19.2 g/l of hemicellulosic sugars (a mixture of mannose, xylose, galactose, arabinose and glucose) with low acetic acid and furfural concentrations. Considering the composition of hydrolysates, it can be concluded that the liquors are potentially useful for fermentative purposes; and that the selectivity towards cellulose degradation could make a process based on the separate utilization of hemicelluloses and cellulose (in the way depicted in Fig. 1) feasible.

Enzymatic hydrolysis of the solid residues from prehydrolysis

As described in previous sections, the dilute acid treatment causes several effects additional to hemicellulose degradation that also enhance the enzymatic hydrolysis, including removal of extractives and structural changes in cellulose. However, owing to the selective dissolution of some fractions, the relative lignin content of the solid residues increases in comparison with that of raw wood (see Table 1), limiting the accessibility to cellulose fibres. So, a counteraction exists between effects enhancing and hindering the enzymatic hydrolysis.

This counteraction of effects could explain the remarkable differences existing in literature on the evaluation of the acid technology as a pretreatment for the

enzymatic hydrolysis. Elander (1994) found that the dilute acid pretreatment improved the enzymatic hydrolysis stage, decreasing the celullase requirements and shortening the reaction times (and so, the size of reactors). Knappert *et al.* (1981) reached cellulose conversions in the range 7.7–74.9% after 48 h in the enzymatic hydrolysis of acid-pretreated poplar wood, and found kinetic improvements when the cellulase complex was supplemented with cellobiase. On the other hand, David *et al.* (1988) found that prehydrolysed *Eucalyptus* wood is a poor substrate for enzymatic hydrolysis.

Figure 2 shows the results obtained when prehydrolyzed pine wood samples (under the conditions of experiments 1 to 6 of Table 1) were used as substrates for enzymatic hydrolysis. Both the kinetics and yields of reaction improved with the severity of the pretreatment. However, the maximum glucose concentration achieved in experiments (5.3 g/l) is too reduced for making suitable fermentation media.

In order to improve the accessibility of enzymes to cellulose, the harsher experimental conditions were selected for the acid prehydrolysis, and a NaClO treatment was carried out before the enzymatic hydrolysis. Table 2 summarizes the experimental conditions used in this step, as well as further information on the residue yield and composition of solid residues, and Fig. 3 shows the generation of glucose when the processed samples were used as substrates for enzymatic hydrolysis. It can be noted that the oxidizing treatments markedly improve the susceptibility of the cellulosic substrates towards enzymatic hydrolysis, leading to a typical hyperbolic kinetic pattern (Holtzapple *et al.*, 1984). The highest glucose concentrations achieved in this set of experiments (from 10 up to 30 g/l) are similar or higher than the concentrations of the carbon sources used in related studies on proliferation of *Phaffia* using this sugar as a carbon source (Johnson & Lewis, 1979; An *et al.*, 1989; Fang & Cheng, 1993; Meyer & du Preez, 1993*a,b*, 1994*b,c*). For example, Johnson & Lewis (1979) found that increased glucose concentrations in the culture media resulted in decreased mass concentrations of AX, the synthesis of this compound being suppressed when the substrate concentration exceeded 40 g/l.

Preparation of fermentative media

Table 3 lists data reported on the growth of *Phaffia* on several carbon sources. Most authors used culture

Table 1. Composition of raw wood, conditions used in acid treatments and composition of reaction products in the prehydrolysis stage

(a) Composition of raw wood (percent, oven-dry basis).
Lignin, 32.0.
Cellulose, 52.7.
Hemicelluloses, 21.1.

(b) Operational conditions used in acid hydrolysis, residue yield and composition of solid residue.
Operational conditions fixed in all the experiments:
Normal boiling temperature.
Liquor/wood ratio = 10/1 g/g.
Other operational conditions:

Exp.	% H_2SO_4	Time, h
1	6.5	2
2	6.5	5
3	6.5	8
4	10	2
5	10	5
6	10	8

(c) Residue yield and composition of phases

		COMP. SOLID PHASE			COMP. LIQUID PHASE		
Exp.	RY[1)]	% L[1)]	% C[1)]	% HC[1)]	S[1)]	AcH[1)]	F[1)]
1	76.7	39.0	34.8	14.2	15.1	1.4	0.1
2	72.3	42.6	47.6	8.8	17.7	1.3	0.2
3	71.2	41.4	46.4	9.6	19.1	1.4	0.3
4	74.8	39.1	47.6	4.3	16.9	2.2	0.2
5	71	42.2	51.9	2.9	18.6	2.3	0.4
6	69.6	41.1	52.0	2.3	19.2	1.6	0.5

[1)] Nomenclature: RY, residue yield (g/100 g); % L, percent of lignin in processed samples (g/100 g processed wood); % C, percent of cellulose in processed samples (g/100 g processed wood); % HC, percent of hemicelluloses in processed samples (g/100 g processed wood); S, sugar concentration in hydrolysates (g/l); AcH, acetic acid concentration in hydrolysates (g/l); F, furfural concentration in hydrolysates (g/l).

media made from commercial sugars, whereas grape and alfalfa juices, residues from corn wet milling and acid peat hydrolysates have also been tried for this purpose. The data on AX concentration varied in a broad range, depending on the carbon source, type and concentration of nutrients and type of strain (wild or mutant). Glucose supports high growth rates, but moderate AX yields (Johnson & Lewis, 1979). For wild strains, the usual carotenoid concentrations vary in the range 200–300 μg/g yeast, with maximum values of 500 μg/g, AX accounting for 40–95% of this concentration (Johnson & An, 1991). As can be seen from Table 3, significantly higher concentrations can be reached using mutant strains (An *et al.*, 1989, 1991; Meyer & du Preez, 1993*a*, 1994*a*,*c*); but in these cases the stability of the micro-organism can be a limiting factor.

Enzymatic hydrolysates of processed lignocellulose have been used for a variety of biotechnological processes (including production of Single Cell Protein, lactic acid, ethanol and acetone-butanol), and can similarly be used for culturing *Xanthophyllomyces dendrorhous* cells. In order to obtain comparative data, culture media were prepared from hydrolysates and from commercial glucose (both containing 15 g glucose/l). The operational conditions used for incubation were chosen according to data from literature: the shaking speed was fixed at 200 rpm, the optimum value reported by Martin *et al.* (1992), and temperature was fixed at 22°C as suggested by Johnson and Lewis (1979). Under these conditions, almost complete substrate depletion was reached in both types of media after 40–60 hours of fermentation.

Figure 4 shows the dynamics of biomass generation in both types of media, as well as the behaviour predicted by the equation of Moraine and Rogovin (1966). As a regression parameter, this equation provides the maximum specific growth rate μ_{max}, which was 0.07 h^{-1} for hydrolysates and 0.14 h^{-1} for the medium containing commercial glucose. This difference suggests that some inhibitors exist in hydrolysates. In fact, the citrate buffer has been cited as an inhibitory agent for *Phaffia* (Johnson & Lewis, 1979). However, the biomass concentrations reached similar levels in both types of media after

Table 2. Experimental conditions used for the oxidizing treatments of solid residues from prehydrolysis, and results obtained

(a) Operational conditions selected for prehydrolysis: those of experiment 6 of Table 1.

(b) Operational conditions used in NaClO treatments
Operational variables fixed in all the oxidation experiments:
Temperature, 60°C.
Reaction time, 1 h
Other operational variables:

Exp.	NaClO conc., mol/l	Liquor/solid ratio, g/g
7	0.60	20
8	0.60	30
9	0.60	40
10	0.80	20
11	0.80	30
12	0.80	40

(c) Residue yield and cellulose content of solid residues from the above treatments:

Exp.	Residue yield, g/100 g	Cellulose content, g/100 g
7	70.0	72.1
8	64.4	74.3
9	52.3	89.5
10	63.2	75.7
11	50.1	95.4
12	49.0	90.2

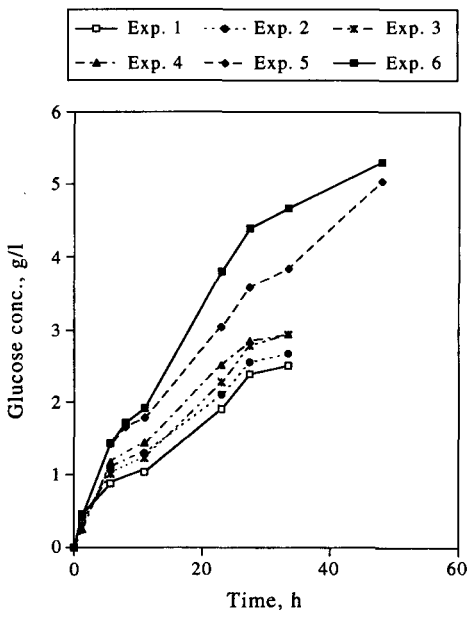

Fig. 2. Glucose generation in the enzymatic hydrolysis of the solid residues obtained in experiments 1 to 6 of Table 1.

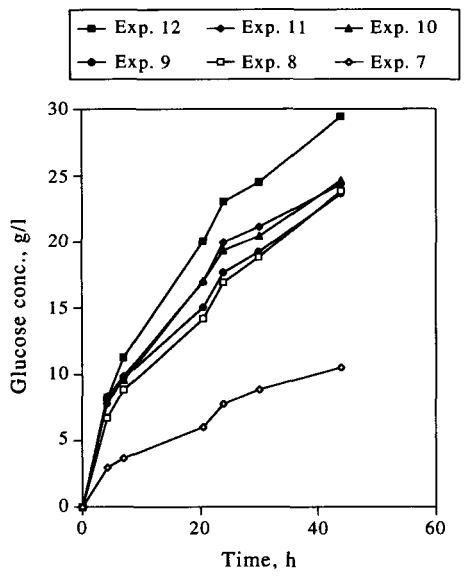

Fig. 3. Glucose generation in the enzymatic hydrolysis of the solid residues obtained in experiments 7 to 12 of Table 2.

Table 3. Results reported on the proliferation of *Phaffia* species growing on several carbon sources

Carbon source or culture medium	Volumetric carotenoid conc. (mg/l)	Mass carotenoid conc. (μg/g yeast)	References
Glucose	1.30–10.12	325–1633	Johnson & Lewis, 1979; Haard, 1988; An *et al.*, 1989; Fang & Cheng, 1993; Meyer & du Preez, 1993*a,b*; Meyer & du Preez, 1994b,c
Cellobiose	0.22–4.10	270–1812	Johnson & Lewis, 1979; Okagbue & Lewis, 1983; An *et al.*, 1989; Fang & Cheng, 1993
Maltose	1.03–5.33	512–1317	Johnson & Lewis, 1979; Fang & Cheng, 1993
Saccharose	1.40–8.4	508–1690	Johnson & Lewis, 1979; Okagbue & Lewis, 1983; Haard, 1988; Fang & Cheng, 1993; Meyer & du Preez, 1994*a*
Xylose	0–1.48	0–819	Johnson & Lewis, 1979; Okagbue & Lewis, 1983; Fang & Cheng, 1993
Arabinose	1.25–9.30	1.4–1014	Johnson & Lewis, 1979; Okagbue & Lewis, 1983; Fang & Cheng, 1993
Glucose + fructose	2.8–29	150–1060	Meyer & du Preez, 1994*a*
Lactose	2.34	1072	Fang & Cheng, 1993
Molasses	0.8–15.3	444–1086	Haard, 1988; Fang & Cheng, 1993
Grape juice	2.0–15.5	120–1510	Meyer & du Preez, 1994*a*
Carbohydrates from peat hydrolysates	—	480–1567	Martin *et al.*, 1992, 1993
Corn wet milling coproducts	0.1–2.6	100–400	Hayman *et al.*, 1995

80–100 h. The values of μ_{max} are similar to those reported by Johnson and Lewis (1979) and Meyer *et al.* (1993*a*), (0.02–0.14 h^{-1}). It can be noted that the concentration of the carbon source leading to the maximum growth rates in the literature (10 g/l) is more favourable than the one selected for this work; but reduced glucose concentrations also result in lower biomass concentrations, limiting the interest of the process.

The above results show that hydrolysates perform well in the bioconversion assays. Martin *et al.* (1992) found that peat hydrolysates were good culture media for biomass production, and suggested that the presence of reaction byproducts (cellobiose) was responsible for this behaviour. It can be noted that in our case the cellobiose concentration is negligible because the medium made from hydrolysates was supplemented with cellobiase, but other fractions (denatured enzymes after sterilization, other components of enzyme concentrates, hemicellulosic sugars remaining in wood and saccharified in the enzymatic step or mineral salts from the solid residue) can also promote the cell growth. These ideas could justify the higher biomass concentration reached for pine wood hydrolysates in relation to commercial glucose media (see Fig. 4). Similarly, Okagbue and Lewis (1984) using culture media made from standard glucose and alfalfa residual juice, found that the higher the juice concentration in the medium, the greater the cell mass produced.

On the basis of the data shown in Fig. 4, it can be inferred that the biomass yield ($Y_{X/S}$) was 0.47 g biomass/g consumed glucose for pine wood hydrolysates, whereas the corresponding value for the commercial glucose medium was 0.40 g/g. These results are in the range reported for *Phaffia* by Meyer and du Preez (1994*a*) (0.23-0.46 g/g, this last range obtained for solutions containing 10 g glucose/l).

Additionally to the biomass concentration, the relative amount and distribution of carotenoids are main factors to be considered in the bioconversion. Figure 5 shows the DAD chromatograms of standard carotenoids and cell extracts. AX was the main carotenoid found in micro-organisms, in relative proportions of 40–60% depending on the type of media and fermentation time. These percentages are typical values for *Phaffia* strains (Johnson & An, 1991). Besides AX, canthaxanthin, echinenone and β-carotene were identified in cell extracts. Two unidentified peaks (termed U1 and U2 in Fig. 5) were also present. Owing to their low practical importance, these compounds (which have not been definitively identified) were integrated using AX as

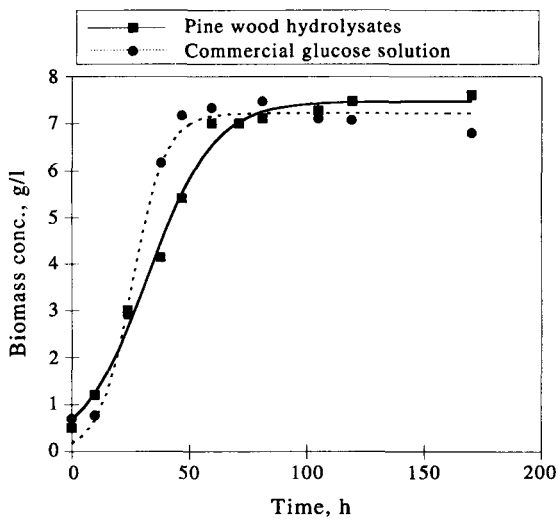

Fig. 4. Dynamics of biomass generation in synthetic or hydrolysate media (dots: experimental results; lines: behaviour predicted by the equation of Moraine and Rogovin).

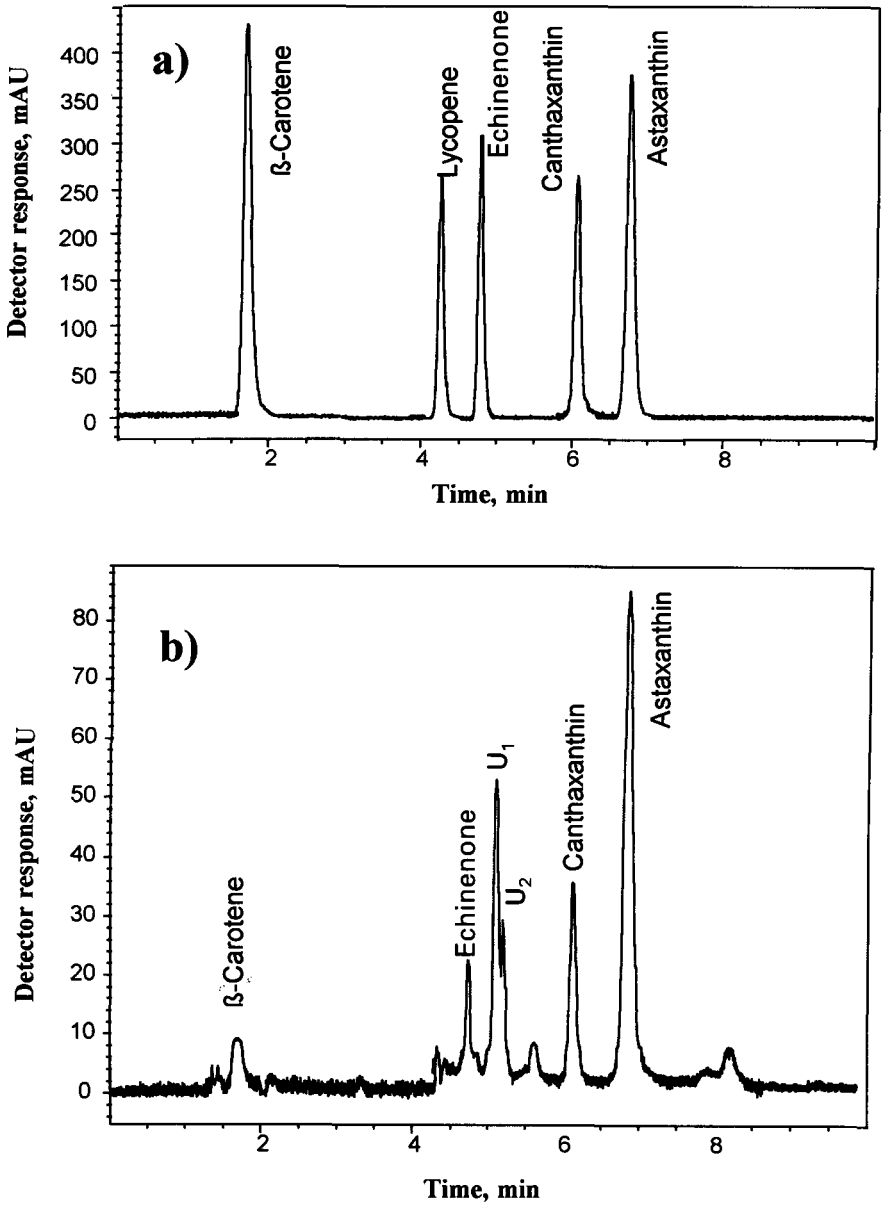

Fig. 5. HPLC chromatograms of: a) standard carotenoids; b) cell extracts.

standard. A comparison of their retention times and spectral features with reported data suggests that they probably are carotenoids like 4-hydroxy-3′,4′-didehydro-β-ψ-carotene (HDC) and 3-hydroxyechinenone, which have been previously identified in *Phaffia* (Johnson & Lewis, 1979; Calo *et al.*, 1995).

Figure 6 shows the volumetric concentrations of AX and carotenoids determined for hydrolysate or synthetic media. The concentration of total carotenoids at the end of assays (1.8–2.1 mg/l) were in the range reported for carotenoid production from glucose (see Table 3). For an adequate evaluation of these data, it can be considered that the utilization of wild strains of *Phaffia* limit both the concentrations and productivities, but have a greater stability in comparison to mutant ones as stated above.

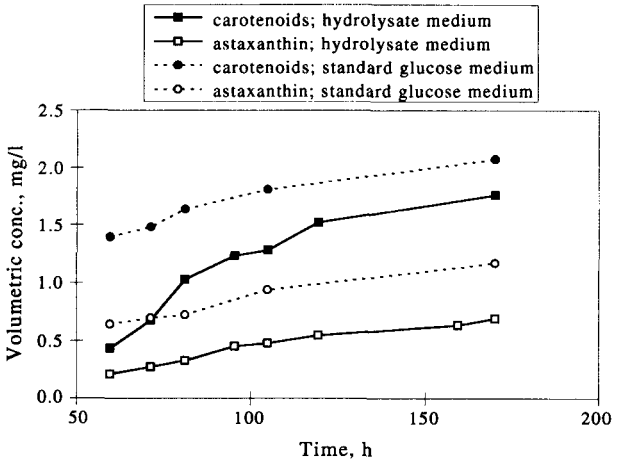

Fig. 6. Volumetric concentrations of astaxanthin and carotenoids obtained in culture media made with commercial glucose or hydrolysates.

CONCLUSION

Wood is a potential source of sugars by means of acid and/or enzymatic hydrolysis. Sequential stages of acid and oxidizing treatments led to a solid residue susceptible to enzymatic hydrolysis, providing a means for obtaining both glucose (up to 30 g/l) and hemicellulosic sugars (up to 19.2 g/l of a mixture containing glucose, galactose, xylose, mannose and arabinose). After supplementation with nutrients, the enzymatic hydrolysates behave as suitable culture media for proliferation of *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*), a red yeast that can be produced at a reasonable growth rate (up to $0.07\,h^{-1}$) with high cell yield (0.47 g biomass/g consumed glucose) reaching concentrations up to 1.8 mg carotenoids/l. The HPLC-DAD analysis showed astaxanthin as the main constituent (accounting for 40–60% of the total carotenoids), with lower proportions of canthaxanthin, echinenone and β-carotene. The results obtained confirm the potentiality of enzymatic hydrolysates for making suitable culture media for astaxanthin production by *Xanthophyllomyces dendrorhous*.

ACKNOWLEDGEMENTS

The authors are grateful to the following institutions and persons: Novo and Hoffman La Roche for the kind supply of enzymes and chemicals; 'Xunta de Galicia' for the financial support of this work (Proj. XUGA38301B95) and Ms. Rocío Rodríguez Fontán for her excellent technical assistance.

REFERENCES

An, G. H., Schuman, D. B. & Johnson, E. A. (1989). Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. *Appl. Environ. Microbiol.*, **55**, 116–124.

An, G. H., Bielich, J., Auerbach, R. & Johnson, E. A. (1991). Isolation and characterization of carotenoid hyperproducing mutants of yeast by flow cytometry and cell sorting. *Bio/technology*, **9**, 70–73.

Andrewes, A. G., Phaff, H. J. & Starr, M. P. (1976). Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. *PhytoChemistry*, **15**, 1003–1007.

Browning, B. L. (1967). In *Methods of Wood Chemistry*. John Wiley & Sons, New York.

Bubrik, P. (1991). Production of asthaxantin from *Haematococcus*. *Biores. Technol.*, **38**, 237–239.

Calo, P., Velázquez, J. B., Sieiro, C., Blanco, P., Longo, E. & Villa, T. G. (1995). Analysis of astaxanthin and other carotenoids from several *Phaffia rhodozyma* mutants. *J. Agric. Food Chem.*, **43**, 1396–1399.

David, C., Fornasier, R., Lejong, W. & Vanlautem, N. (1988). Pretreatment of *Eucalyptus* wood with sodium hypochlorite and enzymatic hydrolysis by cellulases of *Trichoderma viride*. *J. Appl. Polym. Sci.*, **36**, 29–41.

Elander, R. T. & Hsu, T. (1994). Processing and economic impacts of biomass delignification for ethanol production. *Appl. Biochem. Biotechnol.*, **45-46**, 463–478.

Fang, T. J. & Cheng, Y. (1993). Improvement of astaxanthin production by *Phaffia rhodozyma* through mutation and optimization of culture conditions. *J. Ferment. Bioeng.*, **75**, 466–469.

Haard, N. F. (1988). Astaxanthin formation by the yeast *Phaffia rhodozyma* on molasses. *Biotechnol. Lett.*, **10**, 609–614.

Hayman, G. T., Mannarelli, B. M. & Leathers, T. (1995). Production of carotenoids by *Phaffia rhodozyma* grown on media composed of corn wet-milling co-products. *J. Ind. Microbiol.*, **14**, 389–395.

Heikkila, H., Nurmi, J., Rahkila, L. & Toyryla, M. (1992). U.S. Pat. 5.081.026.

Holtzapple, M. T., Caram, S. H. & Humphrey, A. E. (1984). A comparison of two empirical models for the enzymatic hydrolysis of pretreated poplar wood. *Biotechnol. Bioeng.*, **26**, 936–941.

Ivarson, K. C. & Morita, H. (1982). Single-Cell Protein production by the acid-tolerant fungus *Scitalidium acidophilum* from acid hydrolysates of waste paper. *Appl. Env. Microbiol.*, **43**, 643–647.

Johnson, E. A. & Lewis, M. J. (1979). Astaxanthin formation by the yeast *Phaffia rhodozyma*. *J. Gen. Microbiol.*, **115**, 173–183.

Johnson, E. A., Villa, T. G. & Lewis, M. J. (1980*a*). *Phaffia* rhodozyma as an astaxanthin source in salmonid diets. *Aquaculture*, **20**, 123–134.

Johnson, E. A., Lewis, M. J. & Grau, C. R. (1980*b*). Pigmentation of egg yolks with astaxanthin from the yeast *Phaffia rhodozyma*. *Poultry Sci.*, **59**, 1777–1782.

Johnson, E. A. & An, G. (1991). Astaxanthin from microbial sources. *Crit. Rev. Biotechnol.*, **11**, 297–326.

Knappert, D., Grethlein, H. & Converse, A. (1981). Partial acid hydrolysis of poplar wood as a pretreatment for enzymatic hydrolysis. *Biotechnol. Bioeng. Symp.*, **11**, 67–77.

Martin, A. M., Acheampong, E., Patel, T. R. & Chornet, E. (1992). Study of growth parameters for *Phaffia rhodozyma* cultivated in peat hydrolysates. *Appl. Biochem. Biotechnol.*, **37**, 235–241.

Martin, A. M., Acheampong, E. & Patel, T. R. (1993). Production of astaxanthin by *Phaffia rhodozyma* using peat hydrolysates as substrate. *J. Chem. Technol. Biotechnol.*, **58**, 223–230.

Meyer, P. S., du Preez, J. C. & Kilian, S. G. (1993*a*). Selection and evaluation of astaxanthin-overproducing mutants of *Phaffia rhodozyma*. *World J. Microbiol. Biotechnol.*, **9**, 514–520.

Meyer, P. S. & du Preez, J. C. (1993*b*). Effect of acetic acid on astaxanthin production. *Biotechnol. Lett.*, **15**, 919–924.

Meyer, P. S. & du Preez, J. C. (1994*a*). Astaxanthin production by a *Phaffia rhodozyma* mutant on grapejuice. *World J. Microbiol. Biotechnol.*, **10**, 178–183.

Meyer, P. S. & du Preez, J. C. (1994*b*). Effect of culture conditions on astaxanthin production by a mutant of *Phaffia rhodozyma* in batch and chemostat culture. *Appl. Microbiol. Biotechnol.*, **40**, 780–785.

Meyer, P. S. & du Preez, J. C. (1994*c*). Photo-regulated astaxanthin production by *Phaffia rhodozyma* mutants. *System. Appl. Microbiol.*, **17**, 24–31.

Moraine, R. A. & Rogovin, P. (1966). Kinetics of polysaccharide B-1459 fermentation. *Biotechnol. Bioeng.*, **8**, 511–524.

Nelis, H. J. & De Leenheer, A. P. (1991). Microbial sources of carotenoid pigments used in foods and feeds. *J. Appl. Bacteriol.*, **70**, 181–191.

Okagbue, R. N. & Lewis, M. J. (1983). Mixed culture of *Bacillus circulans* wl–12 and *Phaffia rhodozyma* on different carbon sources: yeast-wall lytic enzyme production and extractability of astaxanthin. *Biotechnol. Lett.*, **5**, 731–736.

Okagbue, R. N. & Lewis, M. J. (1984). Use of alfalfa residual juice as a substrate for propagation of the red yeast *Phaffia rhodozyma*. *Appl. Microbiol. Biotechnol.*, **20**, 33–39.

Parajó, J. C., Santos, V. & del Río, F. (1995*a*). Hydrolysis of the cellulosic fraction of *Pinus pinaster* wood. I. Kinetics and product distribution at atmospheric pressure. *Afinidad*, **52**, 162–170.

Parajó, J. C., Alonso, J. L. & Santos, V. (1995*b*). Kinetics of catalyzed organosolv processing of pine wood. *Ind. Eng. Chem. Res.*, **34**, 4333–4342.

Parajó, J. C., Domínguez, H. & Domínguez, J. M. (1996). Production of xylitol from concentrated wood hydrolysates by *Debaryomyces* hansenii: effect of the initial cell concentration. *Biotechnol. Lett.*, **18**, 593–598.

Sedmak, J. J., Weerasinghe, D. K. & Jolly, S. O. (1990). Extraction and quantitation of astaxanthin from *Phaffia rhodozyma*. *Biotechnol. Techniq.*, **4**, 107–112.

Tangeras, A. & Slinde, E. (1994). Coloring of salmonids in aquaculture: the yeast *Phaffia rhodozyma* as a source of astaxanthin. In *Fisheries Processing: Biotechnological applications*. Chapman & Hall, London, UK.

Tjahjono, A. E., Hayama, Y., Kakizono, T., Terada, Y., Nishio, N. & Shiro, N. (1994). Hyper-accumulation of astaxanthin in a green alga *Haematococcus pluvialis* at elevated temperatures. *Biotechnol. Lett.*, **16**, 133–138.

Torrissen, O. J., Hardy, R. W. & Shearer, K. D. (1989). Pigmentation of salmonids-Carotenoid deposition and metabolism in salmonids. *Crit. Rev. Aquatic Sci.*, **1**, 209–220.

Wyman, C. E. (1994). Ethanol from lignocellulosic biomass: technology, economics and opportunities. *Biores. Technol.*, **50**, 3–16.

Yokoyama, A., Izumida, H. & Miki, W. (1994). Production of astaxanthin and 4-ketozeaxanthin by the marine bacterium, *Agrobacterium aurantiacum*. *Biosci. Biotechnol. Biochem.*, **58**, 1842–1844.