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SCREENING OF CAROB BEAN YEASTS. CHEMICAL COMPOSITION OF *SCHIZOSACCHAROMYCES VERSATILIS* GROWN ON AQUEOUS CAROB EXTRACT.

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SUMMARY

An improved extraction procedure for soluble sugars and tannins from carob bean is described. The yeast flora of the carob is rich, with *Saccharomyces* predominant; an isolate of *Schizosaccharomyces versatilis* cultured in the aqueous extract utilizes tannins as well as sugars to give a high biomass and protein yield of good quality.

INTRODUCTION

The carob bean (fruit of *Ceratonia siliqua* L. tree) is a major Greek agricultural product of low commercial value. The ripe pod (pericarp), although rich in water soluble sugars (more than 50%) has a very low protein content (about 6%) (Orphanos and Papaconstantinou, 1969), and contains high levels of total tannins (about 6%) mainly condensed (Tamir and Alumot, 1970; Tamir *et al.*, 1971) which minimizes the nutritional value (Vohra *et al.*, 1966; Tamir and Alumot, 1970). Aqueous carob extract has been used for studies related to fungal protein production (Sekeri-Pataryas *et al.*, 1973; Drouliscos *et al.*, 1976; Macris and Kokke, 1977; 1978), but no studies concerning carob tannin utilization have been published.

Our aim is to improve this agricultural product using yeasts isolated from natural environments. We have investigated the utilization of aqueous carob extract as carbon source for microorganisms as a means of producing tannin-free yeast biomass rich in protein with a balanced amino acid profile. This paper describes: a carob sugar and tannin extraction procedure, a screening of carob bean yeasts on tannin utilization and rich protein biomass production, growth studies and biomass chemical composition of the most effective strain.

MATERIALS AND METHODS

Preparation of aqueous carob extract: A 4 Kg quantity of chopped and deseeded carob pods was mixed with 25 l of deionized water and autoclaved for 30 min at 121°C. The slurry was passed through cheesecloth and the extracted carob pods (spent carob) resuspended in 10 l of deionized water and autoclaved once again. Then the two filtrates were mixed.

Media: a) For isolation of yeasts: malt extract agar, potato dextrose agar, carob extract agar (carob sugars, 2%; $(NH_4)_2SO_4$, 0.33% ; NaH_2PO_4, 0.1% and agar 1.5%) were used. b) Media for batch cultures: 1) M1, M2, M3, M4 (see below) were prepared from an aqueous carob extract supplemented with basal salts and nitrogen source. The carob extract was diluted with salt-water solution to a concentration of total sugars 0.5, 1, 1.5, 2 and 3% (w/v),

so that the composition (g/l) of the media in sugars and salts was:

Components	Media			
	M1	M2	M3	M4*
Carob — sugars	15	5-30	15	15
Carob — tannins	1.4	0.51-3.1	1.5	-
Nitrogen sources	$(NH_4)_2SO_4$,4	NH_4Cl, Urea, $NH_4H_2PO_4$**	$(NH_4)_2SO_4$,4	$(NH_4)_2SO_4$,4
NaH_2PO_4***	0.6	0.2-1.2	0.6	0.6
$MgSO_4.7H_2O$***	-	0.1-0.5	0.3	0.3
KCl	-	-	0.3	0.3

* Carob extract tannins were removed according to the Association of Official Agricultural Chemists (AOAC, 1970).

** The concentration of each nitrogen source was calculated on the base C:N=8:1.

*** The concentration of NaH_2PO_4 and $MgSO_4.7H_2O$ in medium M2 was changed in relation to the total sugars.

2) M5 medium contained (g/l): glucose, 15; $(NH_4)_2SO_4$,4; KH_2PO_4, 1.1; $MgSO_4.7H_2O$ 0.5; $CaCl_2$, 0.15; yeast extract, 1.5; and (mg/l): $ZnSO_4.7H_2O$, 0.06; $CuSO_4.5H_2O$, 0.03; biotin, 0.04; thiamine, 1; pyridoxine hydrochloride, 0.5; nicotinic acid, 0.5; riboflavin, 2.

3) M6 medium contained all M5 componets plus 1.5 g/l of carob tannins.

The carob extract was sterilized by filtration through membrane filter (0.2 μ pore size, Gelman Michigan) and the salt solution by autoclaving (15 min, 121°C) before mixing. The pH was adjusted to 4.5.

Medium M1 was used for primary screening. The selected isolates were cultured in medium M2 to optimize culture conditions (incubation time, initial concentration of total sugars and nitrogen and salt sources). The yeast eventually selected was finally cultured in media M3-6.

Batch cultivation: During primary screening and in experiments for the optimization of the culture conditions, yeasts were grown in 100 ml Erlenmeyer flasks containing 20 ml medium. These flasks were incubated on reciprocal shaker (120 strokes per min). Each experiment was run in triplicate (five flasks per run). The yeast eventually selected was cultivated for growth studies in a culture vessel apparatus as described by Karagouni and Slater (1979). The harvested biomass was collected by centrifugation at 4000 rpm washed several times with distilled water and dried by lyophilization to constant weight.

Isolation of yeasts: Two hundred samples containing carob beans and soil were collected from various areas of the island of Crete. Soil samples were collected from carob storehouses (layer 0-5 cm depth) and from carob tree plantations (layer 5-20 cm depth).

a) Isolation from carob beans: The carob fruit was broken in a sterile Waring blender. A quantity of chopped carob pods was mixed with 10 ml of medium into a petri dish. The petri dishes were divided into groups depending on the used growth medium. Duplicates of each petri dish group were incubated at nine different temperatures (between 25-45°C). The growing colonies were purified by pour-plate and streak-plate methods.

b) Isolation from soil samples: Three methods: dilution plate, syringe inoculation and soil plate were used as described by Barron (1971).

The 700 isolates which obtained were identified according to the classification tables by Lodder (1970) and Barnett *et al.* (1983).

Analytical methods: Total sugars were determined by the method of Dubois *et al.* (1956). Biomass nitrogen and true (Lowry) protein were estimated by the methods Varley (1966) and Gorsuch and Norton (1969) respectively.Nucleic acids were extracted by the method of Delaney *et al.* (1975). RNA was estimated by the method of Gottlieb and Van Etten (1964), and DNA by the diphenylamine procedure as described by Herbert *et al.* (1971) using bakers' yeast RNA and calf thymus DNA (both Sigma Chemical Co.Ltd St.Louis USA) as standards. Non protein nitrogen was estimated by the method of AOAC (1970). Amino acid analysis was carried out by the method of Christias *et al.*(1975). The essential amino acid index (EAAI) was calculated by the method of Oser (1951). Total lipid extraction, saponification of lipids and esterification of fatty acids were carried out by the method of Kull and Jeremias (1972). Biomass B-group vitamins were estimated by the method of Bell (1974). Moisture was estimated by oven drying at 105°C to constant weight. Ash was determined by ignition at 550°C in a muffle furnace. Water soluble total tannins were extracted by refluxing 1 g freeze-dried biomass in 500 ml of distilled water for 1 h. The total tannins in biomass extract, carob extract and culture filtrates were determined by the Folin-Denis colorimetric method (AOAC, 1970), using tannic acid as a standard. To determine caloric content samples were ignited in a Parr oxygen bomb calorimeter at 32 atm oxygen pressure, standardized with benzoic acid tablets.

The criteria accepted for the best yeasts were: 1) Biomass protein content (BPC, % on dry biomass weight), 2) biomass yield (y, gm biomass dry weight/gm carbohydrate consumed), 3) protein yield (yp=(y) · (BPC)), 4) percentage of total tannin reduction of the medium, 5) growth rate, 6) growth temperature.

RESULTS AND DISCUSSION

Extraction of water-soluble carob sugars and tannins: The aqueous carob extract contained 7.3% total sugars and 0.75% total tannins. On this basis carob pods contained about 60.5% and 6.1% on dry weight water extractable sugars and total tannins respectively. Sekeri-Pataryas *et al.* (1973)and Drouliscos *et al.* (1976) used different methods for carob sugar extraction. On the basis of Drouliscos *et al.* (1976) data, from 100 kg deseeded carob pods, about 31 kg and 0.6 kg total sugars and tannins would have been retrieved respectively. Our extraction procedure was more productive in that almost all the water extractable sugars and tannins were retrieved; the method we used is worthy even if we count the extra cost for electricity, since the quantities extracted were much greater. In addition the spent carob (20% of the initial carob pod weight) causes less environmental pollution.

Identification of yeast isolates: During the study up to 700 yeasts were isolated and classified into 30 species belonging to 13 genera (Table I). The *Saccharomyces* group accounted for the 52% of the total isolated yeasts. *S.cerevisiae* appeared at higher frequency (27.4%) than *Kloeckera*(11%), *Candida* (8.2%)and *Schizosaccharomyces* (6.2%).

The fungal flora (Charpentie and Marakis, 1980) and yeast flora of the carob beans is considered to be rich. The invertase negative strains *(S.rouxii, P. farinosa, T. capitatum, S. octasporus)* were isolated from areas close to a vineyard.

Selection of the best isolates: From the 700 isolated examined, forty gave the results presented in Table II. 75% of the forty isolated yeasts gave more than 40% of crude protein while 60% of these strains revealed **y** values between 0.44-0.49. Tannin reduction was significantly higher in cultures

Table I. Identification and distribution of yeasts isolated from carob beans (A), storehouse soil (B) and carob tree plantation soil (C) samples.

Yeast species	Source of isolation A	B	C	D*	E*	Yeast species	Source of isolation A	B	C	D*	E*
Saccharomyces cerevisiae	+	+	+	+	15.4	*Hansenula subpelliculosa*	+	-	-	+	1
" " var.*ellips.*	+	+	+	+	12	*Hanseniaspora valbyensis*	+	+	+	-	1.2
" *rouxii*(osmophilic)	+	-	-	-	1	*Sporobolomyces gracilis*	-	+	+	-	2.6
" *rosei*	+	+	-	+	3.6	*Torulopsis stellata*	+	+	-	+	2
" *chevalieri*	+	+	-	+	1	*Candida guilliermondii*	+	+	-	+	7
" *bisporus*	-	-	+	-	2	" *tropicalis*	+	+	-	+	1.2
" *fermentati*	+	+	-	+	2	*Kloeckera apiculata*	+	+	+	-	8
" *heterogenicus*	+	+	-	+	1.4	" *jensenii*	+	+	-	+	3
" *steineri*	+	-	+	+	2	*Trigonopsis variabilis*	-	+	-	-	1
" *oviformis*	+	+	-	+	3	*Trichosporon capitatum*	+	+	-	-	3
" *acidifaciens*	+	+	+	-	2	*Rhodotorula glutinis*	+	-	-	+	3.2
" *elegans*	+	+	+	+	2	*Schizosaccharomyces pombe*	+	+	+	+	2.2
" *fructuum*	+	+	+	+	4.6	" *versatilis*	+	-	-	+	3
Pichia polymorpha	+	+	+	+	4	" *octasporus*	-	+	+	-	1
" *farinosa*	-	+	+	-	1	*Endomycopsis bispora*	+	+	-	+	2.4
Hansenula anomala	+	-	+	+	1.2						

* D=invertase, ** E=frequency (%)

of carob bean and storehouse soil sample isolates (e.g. *S. versatilis*, *C.tropicalis*, *C.guilliermondii)* than those from carob tree plantation samples. The former microorganisms also gave higher yp=20-25% and y≥ 50% values. The higher **y** value is due to their ability to use carob tannins or other non carbohydrate compounds for biomass production. Possibly these yeasts are adapted to the carob bean environment. Some 18 of the isolates obtained from carob tree plantation soil failed to grow on carob extract (M1) although they were successfully grown on malt extract or potato dextrose agar media. This may be due to the tannin effect.

Table II. Growth parameter mean of 40 yeast isolates.

Crude protein content (Nx6.25):	34-44.7% (on dry biomass)
Biomass yield (y):	0.38-0.56
Protein yield (yp):	12.9-25%
Percentage of tannin reduction:	5-40% (on initial concentration)
Crowth temperature:	30-35°C

Among the forty screening yeasts, 28 with tannin reduction 25-40%, y=0.42-0.56 and yp=20-25% values, were cultured for further studies in M2 under the best possible culture conditions. The microorganism eventually selected was a strain of *Schizosaccharomyces versatilis* which was cultured for growth studies in M3, M4, M5 and M6. From log plots of cell number vs time, yeast growth was greatest in medium M3, with μ_{max}=0.21 h^{-1}. Growth was better on the natural than on the synthetic media. The presence of tannins (initially 1/10 of total sugars) increases biomass production even in synthetic medium M6, and figure 1 shows tannin reduction over 32 h in media M3 and M6. Most tannin reduction occurred during the exponential phase. In *F.moniliforme* the initial tannins did not affect mycelial growth but were not removed (Macris and Kokke, 1977); thus *S.versatilis* is a significantly better organism for biomass production from the carob extract.

Gross composition of *S.versatilis* biomass grown in M3: Table III shows the gross composition of the dry biomass. The high percentage of crude protein (more than 50%) and the low percentage of ash (5.2%) which are both important parameters when biomass is intended for feed or food use, is obvious. Ash content of *S.versatilis* biomass was 28% lower than that reported for *F.moniliforme* (Drouliscos *et al.*, 1976). The true protein N comprised 75% of the total biomass

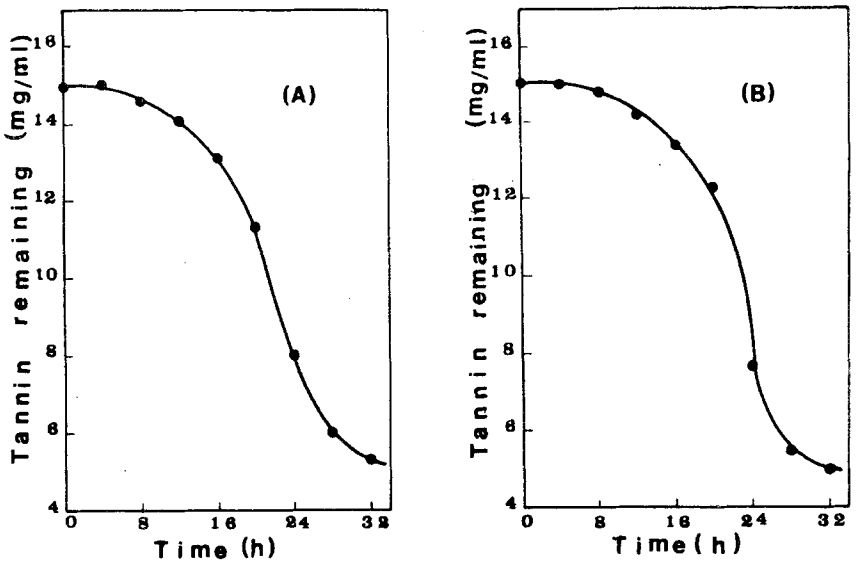

Fig. 1. Tannin utilization in medium M3 (A) and medium M6 (B).

N. The biomass Lowry protein was in agreement with that reported by Macris and Kokke (1977) but significantly higher than that observed by Sekeri-Pataryas *et al.* (1973), Drouliscos *et al.*(1976). The maximum RNA content of *S.versatilis* cells was observed at mid-log, and the minimum at the stationary phase. Minimum RNA level proved comparable or lower to various published data for several yeasts (Trevelyan, 1975; Cooney and Levine, 1975). This is a remarcable feature of *S. versatilis* because after 30-36h of incubation and while RNA and attached tannin contents were minimum, the total amount of single cell protein (mg/ml) was maximum. The percentage of cell attached tannins (0.47%) does not detract from the nutrient quality (Drouliscos *et al.*, 1976). The percentage of the biomass total lipids was about the same as observed in *F. moniliforme* mycelium (Macris and Kokke, 1978) and *S. fragilis* biomass (Delaney *et al.*,1975). The constituent fatty acids of the biomass were: myristic, palmitic, palmitoleic (13%), stearic, oleic (45%) and linoleic (12%). The ash and lipid content of *S. versatilis* biomass do not explain the higher than 0.50 value of y which would be due to the utilization of other carbon substances (i.e. tannins) than the carob sugars.

Table III. Gross composition (%) of *S.versatilis* dry biomass.

Components	%
Total N (TN)	8.16
Non-protein nitrogen	1.85
Crude protein (TNx6.25)	51
Lowry protein	38.80
DNA	1.10
RNA	5.40
Total lipid	7.90
Ash	5.20
Total tannins	0.47
Moisture	3.50

The amino acid profile of *S. versatilis* biomass (Table IV) indicated that the sulfur-containing amino acids were deficient considering the requirements of the growing rat (Smith *et al.*, 1975) and the United Nations FAO/WHO (1965) reference protein. However methionine and cysteine content compared to that of other yeasts was higher. Certain amino acids (i.e. leucine, isoleucine, methionine, cysteine) and total essential amino acid content as also as EAAI were higher than those of *F. moniliforme* and *A. niger* grown on carob extract (Drouliscos *et al.*, 1976).

The caloric content of dry biomass as well as its B-group vitamin are given in Table V. Biomass riboflavin, pantothenic and nicotinic acid and caloric content were higher than those reported by Forage (1978) and Sell *et al.* (1981).

Table IV. Amino acid composition (g/16 g N) of *S. versatilis* biomass.

Phe	4.8	Trp	1.2
Tyr	6.3	Total EAA	56.4
His	1.8	EAAI	85.5
Ile	5.2	Asp	8.8
Leu	8.4	Ser	4.4
Lys	7.6	Glu	13.4
Met	1.8	Pro	4
Cys	1.6	Gly	3.4
Thr	5.1	Ala	4.5
Val	6.3	Total AA	94.9
Arg	6.3		

Table V. B-group vitamin content (µg/g of biomass) and caloric content (KCal/Kg of dry biomass) of *S. versatilis*.

B1	21
B2	64
B6	132
Pantothenic acid	43
Nicotinic acid	161
Biotin	1.2
Folic acid	34
Caloric content	3300 Kcal

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