Enrichment of deseeded carob pod with protein and sucrose or fructose by solid state fermentation

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SUMMARY

The carob bean (fruit of *Ceratonia siliqua,* L.) is an agricultural product of low commercial value. The ripe carob pod (pericarp), although rich in water-soluble sugars, has a very low crude protein content, and contains high levels of tannins, mainly of the condensed type, which minimize its nutritional value. A study of the effect of inoculating deseeded carob pod with mixed culture of *Rhizopus oligosporus* and *Saccharomyces rouxii* or *Saccharomyces cerevisiae* on its protein and sucrose or fructose content, was undertaken. The mixed culture consumed reducing sugars, which resulted in product enriched in sucrose and good quality protein. *S. cerevisiae* hydrolysed the carob sucrose and consumed glucose, thereby producing a product enriched in fructose and yeast protein. Significant amounts of lignocellulose and tannins were also consumed. Both the inoculation reduced the unfavourable effects of the high level of tannins and low protein content on the carob pod nutritional value.

Keywords: Solid state fermentation, deseeded carob pods, mixed culture, *Rhizopus oligosporus, Saccharomyces rouxii,* glucophilic *Saccharomyces cerevisiae*, protein enrichment, sucrose enrichment, fructose enrichment, tannins degradation.

RESUME

Enrichissement de la farine de caroubes en protéines et saccharose ou fructose par fermentation en milieu solide.

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La caroube (fruit du caroubier, *Ceretonia siliqua*, L) est un produit agricole de faible valeur commerciale. La gousse de caroube (péricarpe) riche en sucres est pauvre en protéines. Sa qualité nutritionnelle est encore diminuée par sa forte concentration en tannins. Nous avons entrepris d'enrichir la farine de caroube en sucrose et lactose, respectivement et en protéines de champignons et de levures ou de levures uniquement. Cela a été réalisé par consommation microsélective du glucose et des autres constituants de la caroube par des cultures mixtes de *Rhizopus oligosporus* et *Saccharomyces rouxii*, souches invertase-négatives, ou par culture simple de *Saccharomyces cerevisiae* par fermentation solide. La culture mixte consomme les sucres réducteurs et la caroube obtenue après fermentation est riche en sucrose et enrichie en protéines de bonne qualité. On observe également la consommation d'une importante quantité de ligno-cellulose et des tannins. Les effets nutritionnels défavorables dus aux fortes concentrations de tannins et aux faibles quantités de protéines ont bien été réduits.

Mots clés: Fermentation en milieu solide, farine de caroubes, cultures mixtes, *Rhizopus oligosporus, Saccharomyces rouxii, Saccharomyces cerevisiae* glucophile, enrichissement en protéines, en sacharose, en fructose, degradation des tannins.

INTRODUCTION

Carob bean (fruit of *Ceratonia siliqua*, L. tree) contains 63-90% husk (deseeded carob pod) and 10-37% seeds, depending on the varieties (Mitrakos, 1968; Marakis *et al*, 1987, 1995). The ripe husk, although rich in water-soluble sugars (30-60% of the husk on dry weight basis), has a very low crude protein content (2.5-6.7%), and contains up to 27% tannins (Marakis e*t al*, 1987, 1995; Wursch, 1987), mainly of the condensed type (Tamir *et al*, 1971; Marakis *et al*, 1993), which minimize the nutritional and commercial value of the carob pod (Vohra *et al*, 1966; Tamir and Alumot, 1970).

The nutritional value of carob pod could be improved by eliminating tannins and increasing its protein content. In spite of relevant studies (Drouliscos e*t al*, 1976;

Marakis, 1980; Marakis and Diamantoglou, 1990), such an improvement has not yet been achieved.

Oddo (1928) and Kriaris (1957) failed in extracting sucrose from aqueous carob extract, because carob reducing sugars interfered with sucrose crystallization

Marakis (1992) produced sucrose syrup by microselective consumption (the ability of a microorganism to consume selectively one or some of the components of the substrate) of carob reducing sugars using an invertase-negative mixed culture of *Rhizopus oligosporus* and *Saccharomyces rouxii.* Marakis and Marakis (1995) also succeeded in producing carob fructose syrup by microselective fermentation of glucose by a glucophile strain of *S. cerevisiae.* These studies used aqueous carob extract or carob pod slurry. A direct enrichment of the carob pod with protein and sucrose or fructose by solid state fermentation (SSF) is a new promising approach because 1) the water-soluble (sugars, tannins) and water-insoluble (celluloses, lignin) carob components could be fermented and valorized using suitable microorganism(s), 2) the SSF-system is economically better than the conventional submerged fermentation processes, since reactions simulate the fermentation that occur in nature (Lonsane et al, 1985), and 3) protein synthesis by microorganisms in SSF is a useful alternative for replacing protein sources (soybean cake, fish meal) and improving the content in essential amino-acid of various substrates (Roussos et al, 1994).

Therefore we have studied the effect of inoculating carob husk with a mixed culture of invertase negative *R. oligosporus* and *S. rouxii* or a single culture of the glucophile strain of *S. cerevisiae* in SSF-system on the microselective consumption of glucose or other carob components and the resulting increased concentration in fungal-yeast or yeast protein and either sucrose or fructose.

MATERIAL AND METHODS

MICROORGANISMS

An invertase negative mixed culture of *Rhizopus oligosporus* (Rhol-2) and *Saccharomyces rouxii* (Saro-2) and a glucophile strain of *Saccharomyces cerevisiae* (Sace-1) were used for inoculation. *R. oligosporus* and *S. cerevisiae* strains were isolated from grapes of Santorini island according to Marakis (1980). *S. rouxii* strain has been isolated from carob beans (Marakis, 1992).

CAROB POD VARIETIES

The grafted varieties: g-1, g-2 and H-2 were used because they are 40% richer in sucrose than other Greek carob varieties. Variety g-1 is the richest in fructose (28.1%) among Greek carob varieties (Marakis *et al,* 1995). Our previous study (Marakis *et al,* 1993) has also shown that tannins of g-1 and g-2 varieties lack epicatechin, which reduces microbial growth.

CAROB POD FRAGMENTATION-*KIBBLED* CAROB (HUSK) PREPARATION

The dry carob beans were broken into pieces of about 0.5 cm and the seeds were simultaneously separated using a factory mill.

SOLID STATE FERMENTATION PROCEDURE

The *kibbled* carob was wetted with a solution of $(NH_4)_2SO_4$ (0.5% w/w), and NaH_2PO_4 (0.5% w/w) to a final moisture of 60% that was maintained during the incubation after inoculation with 10^7 spores of *R. oligosporus* + 10^7 cells of *S. rouxii* (mixed culture) or $2x10^7$ cells of *S. cerevisiae* (single culture) per g dry matter. pH was adjusted to 5.0-5.5. The *kibbled* carob and $(NH_4)_2SO_4$ and NaH_2PO_4 solutions were sterilized by autoclaving. The *kibbled* carob fermentation was conducted according to Lambraki *et al* (1994).

ANALYTICAL METHODS

Total water-soluble sugars and individual (sugar profile) were determined according to Dubois *et al* (1956) and Marakis (1992). True protein (protein nitrogen x 6.25) was determined and nucleic acids were extracted according to Delaney *et al* (1975). RNA was estimated by the method of Gottlied and Van Etten (1964) whereas DNA according to Dische (1955), using baker's yeast RNA and calf thymus DNA as standards. Amino acids, water-soluble tannins and ash were estimated, as described by Marakis (1985). Lignin was determined by the methods of Van Soest (1963) and cellulose according to Updergraff (1969).

RESULTS AND DISCUSSION

KIBBLED CAROB FERMENTATION BY MIXED CULTURE (FERMENTATION-A)

The mixed culture of *R. oligosporus* and *S. rouxii* consumed reducing sugars after 36 hours of incubation (Table 1) and produced a fermented carob product (FCP) rich in sucrose (about 45% on dry basis) and enriched in protein (up to 17%) with a good amino acid profile (Table 2). The same microorganisms in submerged liquid mixed culture consumed the carob reducing sugars within 34 hours (Marakis, 1992).

Table 1. Gross composition (% on dry weight) of the fermented carob pod produced by mixed culture of *R. oligosporus* and *S. rouxii* in SSF-system.

Incubat. time ,(h)	Carob varieties	Sucrose (%)	Fructose (%)	Glucose (%)	Protein (%)	Tannins (%)	Cellulose (%)	Lignin (%)
	g-1	42.1	7.6	5.0	4.4	4.4	5.1	8.1
0	g-2	43.4	2.2	0.9	4.7	4.7	6.2	9.3
	H-2	45.0	1.9	0.7	4.8	5.0	9.2	7.2
	g-1	42.6	6.2	3.8	5.5	3.6	4.8	7.7
24	g-2	43.9	1.8	0.6	5.4	4.3	5.8	9.0
	H-2	45.8	1.2	0.6	6.8	3.6	7.2	6.3
	g-1	44.1	Trace	ND	11.5	1.8	4.1	7.4
36	g-2	43.7	ND	ND	10.0	2.3	5.2	8.2
	H-2	46.2	ND	ND	12.5	0.5	6.1	5.8
	g-1	44.4	ND	ND	14.5	1.0	3.1	5.4
48	g-2	44.1	ND	ND	12.0	1.6	4.3	7.1
	H-2	46.2	ND	ND	17.0	0.4	3.1	4.4

ND=Not detected

The amino acid profile of the proteins produced by SSF (Table 2) was similar to that obtained in liquid culture (Marakis, 1992). On the other hand, a tannin content of (0.4% on dry basis) indicated that the activities of the microorganisms degrading tannins were 27 times higher in SSF conditions than in shaken liquid culture (Marakis, 1992).

Table 2. Amino acid composition (g/16 g N) of fermented carob pod (H-2 variety) produced by using mixed culture of *R. oligosporus* and *S. rouxii* in SSF-system.

Phe=4.6	Lys=7.4	Arg=5.5	Glu=12.5
Tyr=5.9	Met=2.0	Trp=1.3	Pro=3.1
His=1.8	Cys=1.7	Total EAA=53.2	Cyl=3.4
Ile=4.9	Thr=4.4	Asp=8.3	Ala=4.4
Leu=7.9	Val=5.8	Ser=4.1	Total AA=89.0

The protein contents (17% on dry fermented product), was higher than that (7%) reported by Kokke (1977). From a nutritional aspect, it is important that ash content remains lower than 5% in the compounded feed. Thus, the FCP protein and ash (4.4%) contents were considered acceptable. Marakis and Diamantoglou (1990) reported that a tannin content (0.4%) did not affect the nutritional quality of FCP and dit not appear to cause a toxicological problem or to depress protein digestibility.

Since lignocelluloses are of low nutritional value and affect true nitrogen digestibility, these substances should be significantly reduced (Malefaki-Perela, 1981). Contents of 3.1% in cellulose and 4.4% in lignin of the FCP of H-2 variety (Table 1) are satisfactory from the nutritional point of view.

KIBBLED CAROB FERMENTATION BY SINGLE CULTURE OF *S. CEREVISIAE* (FERMENTATION-B).

S. cerevisiae Sace-2, under SSF conditions, preferentially used glucose with a simultaneous hydrolysis of sucrose to fructose and glucose, thereby leading to higher fructose concentrations (Table 3). This strain lacks a specific kinase responsible for fructose phosphorylation (Cochrane, 1958). The presence of glucose in carob pod did not allow the enzyme synthesis or enzymatic activation for the fructose fermentation, as in the case of other microorganisms, e.g., *Allomyces macrogynus* (Cochrane, 1958). After 72 h of *kibbled* carob fermentation by *S. cerevisiae*, a FCP containing 28.1% fructose on dry weight (Table 3) and enriched in proteins (21.4%) of good quality (Table 4) was obtained. The same strain, in aqueous carob extract of g-1 variety, hydrolysed the sucrose, while fermenting the glucose within 100 h incubation (Marakis and Marakis, 1995).

Table 3 Gross composition (% on dry weight) of the fermented carob pod (g-1 vatiety) produced by a glucophile strain of *S. cerevisiae* in SSF-System.

Incubation time,(h)	Sucrose (%)	Fructose (%)	Glucose (%)	Protein (%)	Tannins (%)	Cellulose (%)	Lignin (%)
0	42.1	7.6	5.0	4.4	4.4	5.1	8.1
24	39.5	8.6	4.5	5.6	4.0	5.0	8.2
48	21.1	17.8	5.5	7.8	3.1	4.3	8.0
68	1.1	27.5	3.1	16.4	2.8	4.0	7.7
72	ND	28.1	Trace	21.4	0.9	3.5	6.7

ND=Not detected

Table 4. Amino acid composition (g/16 g N) of fermented carob pod (g-1 variety) produced by *S. cerevisiae* in SSF-system.

Phe=4.8	Lys=7.3	Arg=5.7	Glu=14.5
Tyr=5.3	Met=1.9	Trp=1.0	Pro=3.4
His=1.3	Cys=1.6	Total EAA=51.6	Cyl=3.6
Ile= 4.9	Thr=4.8	Asp=8.6	Ala=4.0
Leu=7.1	Val=5.3	Ser=4.4	Total AA=90.1

Thus, under SSF conditions the time for sucrose hydrolysis and glucose consumption was reduced, as compared to that in liquid culture.

The balanced amino acid profile (Table 4), low ash (4.1%) and 5.2% RNA contents of the FCP, encourage protein production from deseeded carob pod.

CONCLUSIONS

In both cases (fermentations-A and -B), the protein content of the FCP was acceptable. From a nutritional point of view, the ash content was also acceptable, since it was lower than that is normally observed in compounded feed (5%). Tannin content decreased to less than 0.4%. This level does not show any toxicological effects and does not depress protein digestibility and utilization. Lignocelluloses

Lignocelluloses were decreased significantly, especially in variety H-2, and their levels in FCP were considered satisfactory from the nutritional point of view. The RNA content of the FCP was also low. Since non-toxic microorganisms were used, the FCP could be used as animal feed or food. Having established an exploitable process for the carob pods, their commercial value will increase. Thus, carob producers will be interested in expansion of carob plantations on barren soils, which are often unproductive for any other type of crop. Thereafter, upgrading of the environment is expected, because barren, rocky and dry regions, where carob tree naturally grows, will become reforested.

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