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SOME CULTURAL CONDITIONS FOR MAXIMUM BIOEXTRACTION OF GRAPEFRUIT WASTE PECTIN

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ABSTRACT

Bioextraction of pectin from grapefruit waste by *Kluyveromyces marxianus* was markedly influenced by the age of seed culture, inoculum size and medium volume/ flask (aeration), as well as the pH value of the fermentation. Thus, 24h old seed culture at inoculum size of 4% and 60 ml medium/250ml Erlenmeyer flask and pH value of 6, were responsible for maximum pectin extraction (about 99% of that originally contained in GFW), by the tested yeast. The culture filtrate was found to have some hydrolytic enzymes. The major enzyme activities resided in endopolygalacturonase followed by protopectinase. While cellulase activity was nearly absent and very low amylase activity was also detected. On the other hand, glucoamylase activity was not detected. The characterization of both microbiologically and chemically extracted pectin samples indicated that the former had higher percentages of methoxyl groups, higher degree of estrification and higher free carboxyl groups, but lower values of galacturonic acid and thus possesses superior qualities to chemically extracted pectin

Keywords: Bioextraction – Pectin – *Kluyveromyces marxianus* – Grapefruit waste

INTRODUCTION

Protopectin is the water-insoluble parental substance of pectin found in plant tissues; it yields water-soluble pectin upon restricted depolymerization. Pectin-releasing or pectin solublizing enzymes are also called protopectinases[1]. Several protopectinases from different microbial origins were isolated and characterized [2]. Pectin is widely used as a texturizer and stabilizer in a variety of foods and other industries. Despite its availability in a large number of plant species and so in acids under conditions of pH, time and temperature chosen to maximize yield while retaining quality. Materials, handling equipment pectin are very limited [3].Commercial extraction of pectin is usually accomplished with mineral and cost have also to be considered [4]. Both yield and quality are important factors in determining the suitability of the extraction method [5].

In a previous paper, the authors[6] characterized some cultural conditions that maximize protopectinase activity and pectin bioextraction from grapefruit waste in cultures of *Kluyveromyces marxianus*. The bioextraction of pectin was influenced by yeast extract, peptone, glucose, time of substrate addition and its levels, as well as incubation period. In this paper we describe the results of experiments to attain a better bioextraction of pectin and protopectinase activity from grapefruit waste by *K. marxianus* through the age and size of seed culture, solid/liquid ratio and initial pH value. The activity of some hydrolytic enzymes in the culture filtrate of the yeast was tested. Finally, the characteristics of the extracted pectin were also considered.

MATERIALS AND METHODS

GRAPEFRUIT WASTE

The waste was obtained from the local market and dried in an oven at 60°C for constant weight. The dried waste was finely ground in a Wiley mill and passed through a 60 gauge mesh to give a homogenous powder for routine work.

MICROORGANISM AND CULTIVATION

K. marxianus 70343 DSM (Deutch Sampling Von Microorganismen) was maintained on agar slants containing 2 % glucose, 0.2% pectin and 0.1 yeast extracted at pH 5[7]. For the seed culture, standard inoculum (4% yeast suspension/100 ml medium) of 24h old culture of the yeast was allowed to grow in a medium of 2% glucose, 0.4% peptone and 0.2% yeast extract, pH 5 at 30°C for 24h under shaking conditions. The yeast was cultivated in 50ml

grapefruit pectin was characterized by the determination of free carboxyl groups by titration against standardized sodium hydroxide, methoxyl content [19], degree of esterification as percentage of methoxylated carboxyl groups to total carboxyl groups, relative viscosity of 5ml of pectin solution (0.5%) at 30°C using an Ostwald viscometer and specific viscosity which was derived from the relative viscosity. The galacturonic acid and sugar constituents were also determined[20,21].

STATISTICAL ANALYSIS

Each treatment was carried out in triplicate and the results obtained throughout this work were subjected to a one way analysis of variance. The mean were compared by Fisher' s test at a significance level of $P< 0.01$.

RESULTS AND DISCUSSION

EFFECT OF AGE OF SEED CULTURE

The data given in Fig.1 show that pectin bioextraction and protopectin solubilizing enzymes were highly influenced with the age of *K. marxianus* seed culture. One day old seed culture (24h) exhibited maximum pectin extraction (34.86±0.12 %) and protopectinase activity (10.30±0.10 U/ml). Seed cultures of younger or older ages showed lower efficiencies. These results are comparable with those reported by some workers [22] by the same organism using beet pulp waste as a source of pectin.

The analysis of variance indicated that the variations with the different age of seed culture in extracellular protein, pectin bioextraction, growth and protopectinase activity were highly significant ($P<0.001$).

SIZE OF INOCULUM

The results (Fig.2) revealed that increasing the quality of inoculum (which may be considered as enzymes source) led to an increase in pectin yielding 93.45% of the total pectin present in grapefruit waste, and protopectinase activity (10.33±0.11 U/ml) when a size of 4% inoculum was used. Any further increase in the size of inoculum was accompanied with a decrease in yield of bioextracted pectin and protopectinase activity. A larger size of inoculum (about 10%) of the same experimental yeast was reported to fulfill maximum pectin extraction from beet pulp [22]. These results indicate that size of inoculum depends on the substrate used and the other fermentation conditions and not on the test organism used as a source for the same degrading enzymes. The inoculum size was also reported to be an important factor in other microbial fermentation of agroindustrial wastes [23].

The analysis of variance indicated that the variations with the different size inoculum in extracellular protein, pectin bioextraction, growth and protopectinase activity were highly significant ($P<0.001$).

EFFECT OF MEDIUM VOLUME /250 ML ERLENMEYER FLASKS

The influence of the volume of culture medium /flask (aeration) on the bioextraction of pectin and protopectinase produced in the culture (Fig.3) indicated that 60ml medium/ 250ml Erlenmeyer flask and shaking conditions of 200 shakes/min offered aeration conditions that favor the highest production and/or activity of pectinase that solubilize the non-soluble grapefruit protopectin. Thus maximum bioextracted pectin (36.06±0.01%) and protopectinase activity (12.87±0.12 U / ml) were obtained at 60ml/flask. Higher volumes showed an adverse effect on pectin extraction and protopectinase activity. It was indicated that medium size/flask had a great influence on the activity of lignin degrading enzymes [24].

The analysis of variance indicated that the variations with the different medium volume in extracellular protein, pectin bioextraction, growth and protopectinase activity were highly significant ($P<0.001$).

EFFECT OF pH

The data (Fig. 4) indicted that the optimum pH was around 6 for optimal pectin yields (36.95± 0.01% about 99% of that contained in grapefruit waste) and protopectinase activity (13.19±0.01 U/ml). It was reported that most polygalacturonases have optimal pHs in the weakly acidic region [7]. It was also indicated that pH 6 was optimal for pectin bioextraction from beet pulp [25]. Therefore, the slightly acidic nature of water is suitable for the enzyme reaction. Other workers [26] indicated that pH below 7 was optimum for protopectinase production and expression by *Geotrichum klebahnii*. The aforementioned results indicated that *K .marxianus* efficiently produce protopectinase

together with a relatively high degree of esterification in MEP sample, is partially responsible for the strong water binding capacity of the grapefruit. High content of acetate was reported on pectin from other sources [32].

Table 2. Composition and some characteristics of the chemically extracted pectin (CEP) and microbially extracted pectin (MEP) samples.

Pectin samples	Moisture content	Solubility	pH of 4%solu.	Ash content	Methoxyl (%)	Degree of estrification (%)	Free carboxyl (%)	Galacturonic acid (%)	Viscosity (%)
CEP	3.4	Complet ely	4.65	0.52	6.2	61.9	3.2	85	1.4
MEP	6.5	Soluble	4.32	1.71	9.9	88.3	4.2	71	1.2

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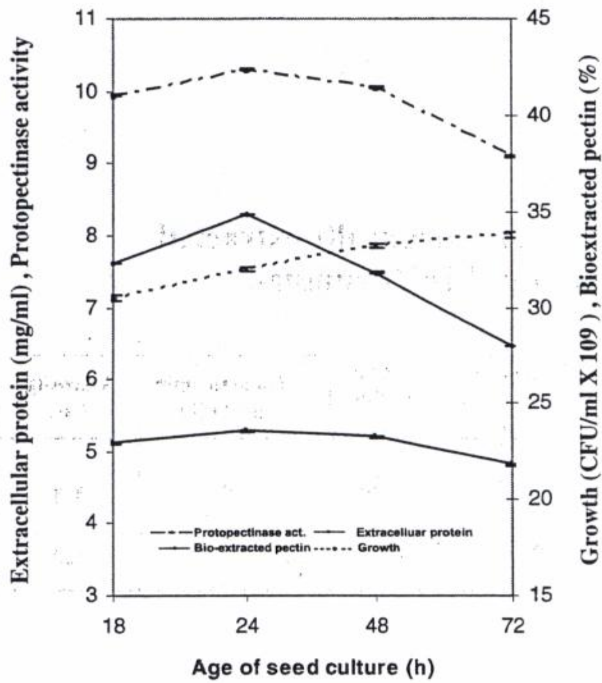

Fig. 1: Pectin bioextraction by *K.marxianus* as influenced with the age of seed cultures

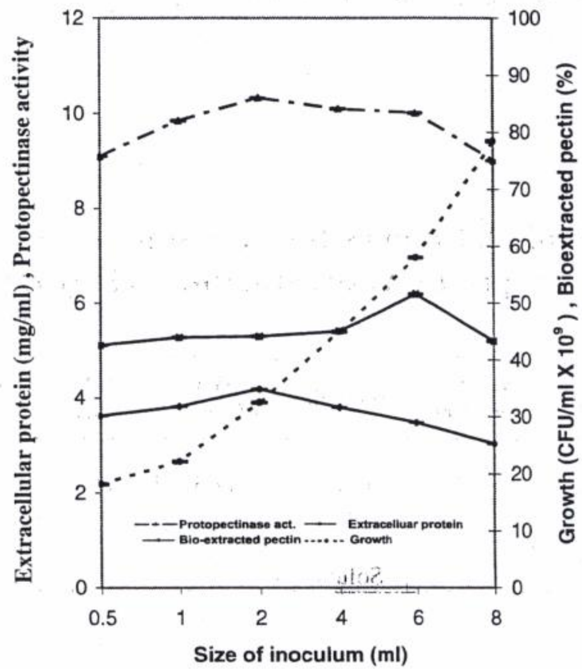

Fig. 2: Pectin bioextraction by *K.marxianus* as influenced with the size of inoculum.

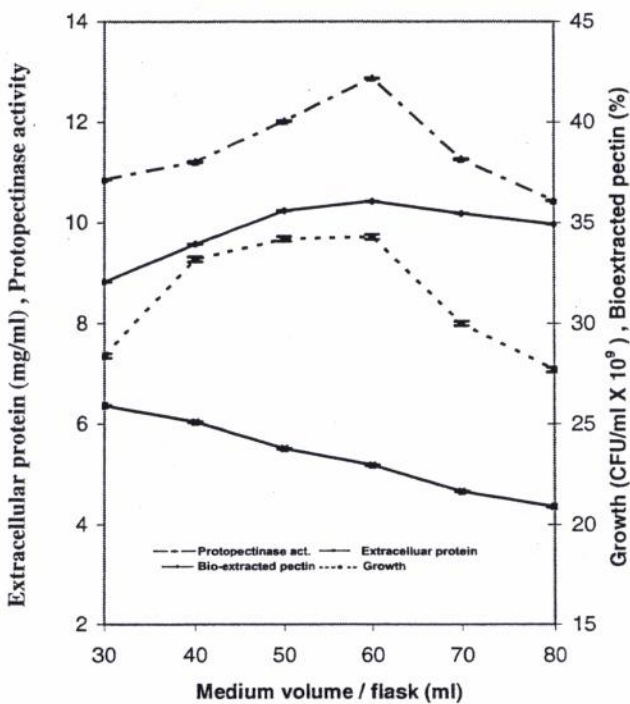

Fig. 3: Pectin bioextraction by *K.marxianus* as influenced with medium volume (liquid/solid).

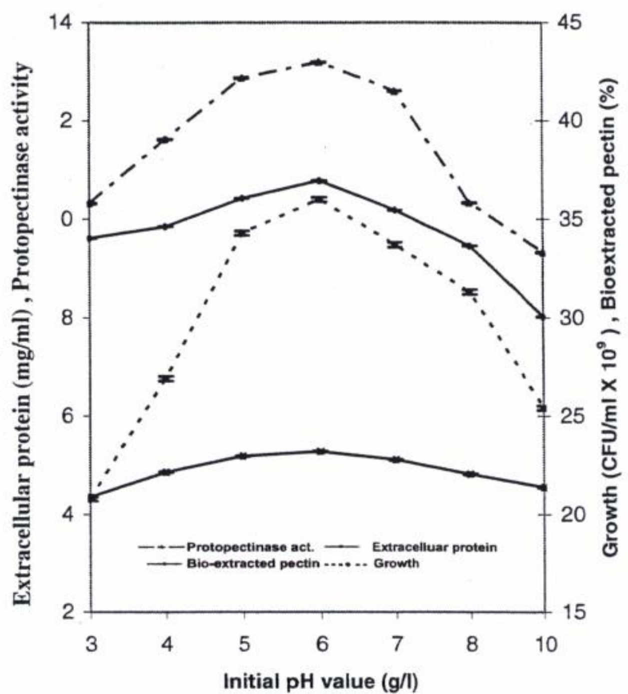

Fig. 4: Pectin bioextraction by *K.marxianus* as influenced with pH value of the medium.

with high activity under simple fermentation conditions and can solublize about 99% of the pectin content of grapefruit., The remaining 1% of pectin may be metabolized by the test organism. These optimal conditions were (%): grapefruit waste 8; peptone, 0.4; glucoses 1, pH 6, 60ml medium dispensed in 250 ml Erlenmeyer flask, inoculated by 4% of 24h old seed culture. Incubation was done at 30°C under shaken conditions (200 shakes/min) for 18 h.

The analysis of variance indicated that the variations with the different pH value in extracellular protein, pectin bioextraction, growth and protopectinase activity were highly significant ($P<0.001$).

HYDROLYTIC ENZYMES IN THE CULTURE FILTRATE OF K. MARXIANUS

The activities of enzymes thought to be present in the supernatant (filtrate) of the experimental yeast were examined (Table 1). These enzymes were endopolygalacturonase, cellulase, cellobiase, amylase and glucoamylase

Table1. Determination of some hydrolytic activities present in the culture supernation of *Kluyveromyces marxianus*

Measured activity	Substrate used	Activity
Protopectinase	Protopectin (grapefruit)	6.2 U/ml
Endo-polygalacturonase	Pectin (citrus)	0.9 U/ml
CMC'ase	Carboxymethylecllulose	0.07 U/ml
FP'ase	Filler paper strip (whatmann No.1)	-ve
Cellobiase	Cellobiose	0.4 U/ml
Amylase	1% soluble starch	0.01 U/ml
Glucoamylase	1% soluble starch	-ve

production of polygalacturonase activity by . It was found that an endopolygalacturonase activity accompanied the protopectinase activity. Both activities were found to be major ones in the studied cultures of the test organism. The other *Kluyveranyces* species, as well as other yeasts, has also been reported by many workers [27,28,26]. In the present work cellulase activity was nearly absent in the culture filtrate of *K. marxianus* since filter paper hydrolase was not detected and a very faint carboxymethcellulase activity was measured. These results are similar to those reported by some workers on the absence of cellulase activities in yeast cultures producing protopectinase enzymes [7,29]. In the present work a very low amylase activity was also detected in the culture filtrate of K. marximnus. The presence of different hydrolytic activities together with pectinase in K. marxianus cultures was also reported [30].

SOME CHARACTERISTICS OF THE GRAPEFRUIT PECTIN EXTRACTED CHEMICALLY AND MICROBIOLOGICALLY

As both yield and quality are important factors in determining the suitability of the extraction method, the microbiologically extracted pectin (MEP) was chemically characterized in comparison with a chemically extracted pectin (CEP) sample. The results (Table 2) revealed that both pectin samples proved to contain relatively low galacturonic acid residues (85%, 71% for CEP and MEP, respectively). Compared to pectin preparations from other sources, it was found that onion skin pectin has 96-96.6% galacturonic acid [11]. However, the present results are comparable with that of lemon (commercial) and citrus pectin that contained 85 and 80.3%, respectively [8]. Also beet pulp pectin contained low galacturonic acid, 74.4% for CEP and 82% for MEP [31]. This may also point to a property in favour of the microbial pectin bioextraction approach in their experiments similar to the present results. It has been found that citrus pectin extracted by fermentation has a lower galacturonic percentage compared to the acid extracted one [8]. MEP is also characterized by its high content of methoxyl and hence high degree of esterification. It was also reported that, when the methoxyl group and galacturonic acid contents of the substrate increase, the molecular weight of the reaction products increase [27]. In addition it was noticed that the molecular weight of pectin has a marked influence on its gellifying ability and for this reason pectin having a large molecular weight is desirable. MEP and CEP showed moderate values of free carboxyl groups reaching 4.2 and 3.2%, respectively. This,

aliquots/250ml Erlenmeyer flasks of the following medium: Grapefruit waste 8%, glucose 1.0%, peptone 0.4%, pH 6, and inoculated with 4% inoculum size (85 x 10^8 yeast cells/ml) of 24h old seed culture, incubated at 30°C for 18h under shaking conditions (200 shakes /min, amplitude 7cm). These favored maximum pectin bioextraction and the highest protopectinase activity recorded by *K. marxianus* [6].

EXTRACTION AND DETERMINATION OF PECTIN SUBSTANCES

At the end of the fermentation time, the filtrate was separated by centrifugation at 4000 rpm for 20 min and poured in 3 volumes of ethanol. The precipitated pectin was collected by centrifugation, washed with ethanol, dried under vacuum at 37°C and then weighed accurately[8]. The pectin constituent of GFW was also extracted and estimated chemically[9].

MICROSCOPIC DETERMINATION OF YEAST GROWTH

This was determined by counting the number of cells in a Thoma chamber (0.05mm length and 0.1 mm depth). The number of cells was calculated by the following equation: $N=XF\ 10^6$, where N= number of cells, X= mean number of cells counted in four squares of Thoma chamber and F= dilution factor. The results were presented as colony forming units per ml culture (CFU/ml), since budding or attached cells were counted as one unit[10].

AGE OF SEED CULTURE

Portions (50ml) of seed culture medium were dispensed in 250 ml Erlenmeyer flasks, sterilized and mixed with standard inocula of the tested organism (2ml). The culture flasks were then shaken at 30°C for different periods (18, 24, 48 and 72h). From each of these different age cultures, 2 ml (85x 10^8 yeast cells/ml) were transferred as inocula into the fermentation medium, as previously described.

SIZE OF INOCULUM

Different enzyme source concentrations were introduced to the substrate by changing the size of inoculum from 0.5 to 8 ml (85 x 10^8 cells/ml) per flask of one day old (optimal age) seed cultures.

CULTURE VOLUME / FLASK (AERATION)

This was carried out by varying the medium (best recorded) volume /250 ml Erlenmeyer flask, the tested volumes ranged from 30 to 80 ml/flask. After sterilization each flask received 4% inoculum size (optimal size) of 24 h old seed culture.

pH RELATIONS

Aliquots of bioextraction medium (60ml/flask, optimal volume) were initially adjusted either with 0.1N HCl or NaOH to pH values ranging from 3 to 10. After 18h of incubation the necessary analyses were carried out.

ENZYMES ASSAYS

Protopectinase activity was determined by measuring the amount of pectic substance liberated by carbazole sulfuric acid method [11]. The pectin concentration was measured as D-galacturonic acid from the standard assay curve with D-galacturonic acid. A unit of enzyme activity (U) was defined as the amount of enzyme required to produce one µmole of D-galacturonic acid min^{-1}, under the tested conditions. Polygalacturonase (pectinase) activity was determined by measuring the reduction in the viscosity of buffered pectin solution (0.0M acetate buffer, pH 4.05) using Ostwald viscometer [12]. Cellulase activities were determined as carboxymethylcellulase and filterpaper hydrolase [13]. The released reducing sugars were measured colourimetrically [14]. One unit of activity was defined as the amount of enzyme producing one µmole of glucose min^{-1}, under the standard assay conditions. Cellobiase activity was assayed according to the method described by Grous *et al* [15]. One unit of activity was defined as the amount of enzyme producing one µmole of glucose min^{-1}. Amylase activity was examined according to the method described by Bergmann *et al* [16]. Both Somogyi reagent [17] and Nelson reagent [18] were used, and the released sugar was estimated colourimetrically using maltose as a standard. One unit of activity was defined as the amount of enzyme producing one µmole of maltose min^1, under the tested conditions.

CHARACTERIZATION OF EXTRACTED PECTIN

The microbially and chemically extracted