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Isolation, Screening, and Characterisation of Flocculating and Pectinase Producing *Kluyveromyces* Strains

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Summary

Eleven strains of Kluyveromyces marxianus were studied to select the best producers of extracellular endo-polygalacturonase (endo-PG), using glucose and lactose as carbon source. Flocculation ability was also assayed. Four out of eleven strains produced more enzyme when glucose was used as substrate. Only one strain was a better endo-PG producer when lactose was used as carbon source, although producing less enzyme than the best performing strain grown on lactose. The best enzyme production was obtained for strain K. marxianus CH4-1 when grown on glucose. In all cases, as a consequence of a lower rate of substrate consumption, enzyme productivity was between two- and three-fold lower when yeasts were grown on lactose. It was also shown that all strains formed flocs only when grown on lactose; on glucose the cells remained powdery.

Keywords: Kluyveromyces marxianus, endo-polygalacturonase, flocculation

Introduction

Production of chocolate requires an initial natural fermentation of the beans of the tree *Theobroma cacao*. In Brazil, approximately 800 kg of beans are fermented in wooden boxes and yeasts dominate the fermentation for the first 24 hours when most of the pulp surrounding the beans is degraded (*1*). Cocoa pulp comprises 14 % sugars and 1.5 % pectin. Four pectinolytic yeast species, which all produced only endopolygalacturonase (endo-PG), were isolated from cocoa fermentations and *Kluyveromyces marxianus* isolates were the best enzyme producers (*2*). Endo-PG has strong activity in reducing the viscosity of cocoa pulp but the pectinolytic yeasts including *K. marxianus* do not produce enough endo-PG during cocoa fermentation to ensure rapid loss of pulp, which is essential to produce the best quality chocolate. By adding purified enzyme potentially poor fermentations (with too much pulp) could be converted into good fermentations (unpublished data). Endo-PG also finds valuable applications in the processing of fruits or fruit juices. Due to the industrial importance of endo-PG there is a need to develop a more efficient system of enzyme production.

Until now, endo-PG has been obtained from *Aspergillus niger* fermentations. This fungus secretes a wide range of enzymes and other metabolites which makes for rather complex downstream processing to yield the purified enzyme (*3*). The fact that about 90 % of proteins secreted by *K. marxianus* under anaerobic conditions are polygalacturonases (*4,5*) makes this microorganism a very attractive system for polygalacturonase production. In addition, *K. marxianus* can ferment lactose, the carbohydrate present in cheese whey. Since cheese whey is available in large amounts due to the problems associated with its disposal, it presents a very attractive substrate for industrial fermentations.

Eleven strains of *K. marxianus* isolated from cocoa fermentations were studied, using synthetic media with glucose and lactose as carbon source, in order to select a strain that both produces larger amounts of extracellular enzyme and has the ability to flocculate. This latter characteristic is of particular importance, since increased

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productivity can be obtained by using high cell density flocculation bioreactors for endo-PG production.

Material and Methods

Yeast isolation and identification

Samples of cocoa fermenting mass were taken every 6 hours over seven days in sterile flasks and diluted for plate counting (*6*). Yeasts were isolated on malt extract agar (pH = 3.5) and TYGKCC medium (*7*) which consisted of tryptone 5.0 g, yeast extract 5.0 g, D-glucose 10.0 g, K_2HPO_4 1.0 g, $CaCO_3$ 1.0 g, and cocoa seed pulp 10 mL per 1L of solution. Yeast species were identified by standard methods (*8,9*). All strains were maintained at 4 °C on agar slants containing: yeast extract (Merck), 3.0 g; peptone (Merck), 5.0 g; glucose, 10.0 g; malt extract (Merck), 3.0 g; and agar 15.0 g per 1L of solution.

Screening for polygalacturonase activity

All isolates were screened for polygalacturonase activity on plates. The volume of 0.1 μL of fresh cells were inoculated on medium MP5 (*2*) and incubated for 48 h at 30 °C. After incubation the plates were flooded with a 1 % (w/v) aqueous solution of hexadecyltrimethylammonium bromide (cetrimide, Sigma, St. Louis, MO). This reagent precipitates intact polygalacturonic acid in the medium and thus clear zones around the colony in an otherwise opaque medium indicate degradation of polygalacturonic acid.

Media and culture conditions for strain selection

Selection of the strains with higher endo-polygalacturonase activity and with flocculation ability was done in the medium modified from Patching and Rose (*10*) containing: 10.0 g glucose or 10.0 g lactose, 1.0 g yeast extract, 3.0 g $(NH_4)_2SO_4$, 4.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$ and 0.25 g $CaCl_2$ per 1 L of solution, adjusted to pH = 5 with NaOH.

Yeast growth was conducted in 250 mL closed, round flat-bottomed flasks with 100 mL of sterilised (120 °C, 20 min) medium. The flasks were inoculated and incubated on an orbital shaker at 150 rpm at 30 °C.

Analysis

Biomass was estimated by a turbidimetric method from a reference calibration curve (one for each strain). Glucose and lactose were determined by Miller's method (*11*). Total proteins were measured using a protein assay kit (Sigma) based on Lowry method after dialysis of supernatant with a membrane having 25000 Da cut-off. For PG activity measurement, the culture was centrifuged, filtered and dialysed with a membrane having 14000 Da cut-off. Activity was determined by measuring the release of reducing groups as described by Schwan and Rose (*12*). Polygalacturonase activity was expressed as μmole galacturonic acid equivalents released per litre and per hour. Flocculation ability was evaluated by microscopic observation of culture samples during the batch culture.

All analyses were performed in duplicate and the corresponding variation coefficients were determined. Values given are those obtained at the end of the exponential phase.

Results and Discussion

Screening of pectinolytic activity

A total of 453 yeast isolates were identified to species level. Most of the yeast strains were found during the first 36–48 h of cocoa fermentation by which time the pulp had been degraded. Pectinolytic activity based on size of the halo on MP5 was determined in all isolates. One hundred and eight isolates showed some pectinolytic activity and the diameter of the halo varied from 2 to 34 mm. Although *Kluyveromyces marxianus* was the best producer, the size of halos varied amongst the isolates ranging from 28 to 34 mm.

Screening of polygalacturonase-producing Kluyveromyces marxianus strains

Fifty-six isolates of *Kluyveromyces marxianus* were selected for growth on liquid medium containing 1 % of glucose and 1 % of pectin. Ten isolates (CH4-1, CH5-1, CH5-3, CH5-4, CH9-1, CH1-1, CH0-1, CH8-1, CH2-2, CH8-9) showed the best results on both culture media for PG secretion and biomass production. These results were comparable with those found for *K.marxianus* CCT 3172 (*2,12*).

Selection of the best endo-polygalacturonase producing strains

The ten isolates described above as well as the *K. marxianus* CCT 3172 strain were tested both for their ability to produce endo-polygalacturonase using lactose as carbon source as well as for flocculation capacity in batch fermentations (see Media and culture conditions for strain selection).

Batch fermentations using glucose (Fig. 1) and lactose (Fig. 2) as carbon source, for the strain *K. marxianus* CCT3172 are presented in Figs. 1 and 2, respectively.

Fermentation values obtained at the end of the exponential growth phase are presented in Table 1.

For this strain, no significant differences were observed between growth on glucose and on lactose with respect to biomass, proteins after dialysis concentration or endo-PG activity. However, in terms of rate of substrate consumption, when lactose was used, total consumption occurred after 40 hours, while in the case of glucose only 13 hours were needed for substrate exhaustion. As a consequence, endo-PG productivity was 3 times higher when glucose was used as substrate. It was also confirmed that polygalacturonase secretion was cell growth related, regardless of the carbon source. No increase in activity was observed during stationary phase. Fermentations were carried out for all strains and the fermentation profiles were similar to that obtained for *K. marxianus* CCT3172. The most important results are presented in Table 2.

Taking into account that the variation coefficient for biomass determination is 5 % and analysing the data presented in Table 2, the results show that for all strains,

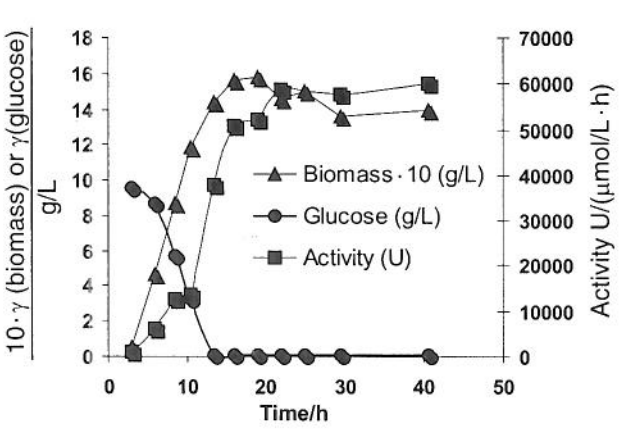

Fig. 1. Batch fermentations using glucose as carbon source

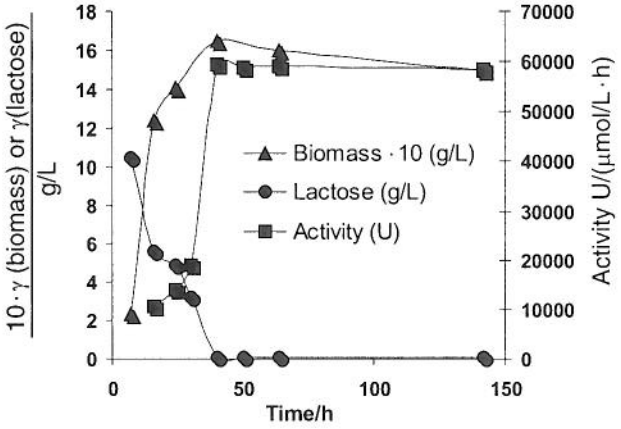

Fig. 2. Batch fermentations using lactose as carbon source

Table 1. Biomass, total protein and protein after dialyis concentration, endo-PG activity and productivity, obtained at the end of the exponential phase using glucose and lactose as carbon source with strain CCT3172

	Carbon source	
	Glucose	Lactose
Biomass concentration/(g/L)	1.6	1.7
Total protein concentration/(mg/L)	—	184.5
Protein after dialysis concentration/(mg/L)	24.0	35.9
Endo-PG activity $\cdot 10^{-3}$/(mole/(L·h)) or (U)	60	59
Endo-PG productivity $\cdot 10^{-3}$/(U/h)	4.6	1.5

Table 2. Biomass concentration, endo-PG activity and endo-PG productivity for ten strains, with glucose or lactose as carbon source

	γ(biomass)/(g/L)		Endo-PG activity $\cdot 10^{-3}$/(U)		Endo-PG productivity $\cdot 10^{-3}$/(U/h)	
	Carbon source		Carbon source		Carbon source	
Strains	Glucose	Lactose	Glucose	Lactose	Glucose	Lactose
CH4-1	1.3	1.8	91	45	6.5	1.9
CH5-1	1.4	1.8	73	61	5.2	2.5
CH5-3	1.4	1.9	66	69	4.7	2.9
CH5-4	1.3	1.8	48	55	3.4	2.3
CH9-1	1.4	1.7	76	56	5.4	2.3
CH1-1	1.6	1.7	52	51	3.7	2.1
CH0-1	1.5	1.7	56	52	4.0	2.2
CH8-1	1.5	1.7	55	41	3.9	1.7
CH2-2	2.0	1.8	57	51	4.1	2.1
CCT 3172	1.6	1.7	60	59	4.6	1.5

with the exception of strains CH1-1 and CCT 3172 where biomass formation was similar regardless of carbon source and strain CH2-2 where higher biomass yields are obtained for glucose, biomass formation was higher when lactose was used as carbon source.

With respect to endo-PG production (the variation coefficient for activity quantification was determined as 10 %), the results shows that for four strains (CH4-1, CH5-1, CH9-1 and CH8-1) the enzyme activity was higher when glucose was used as substrate. A particular

reference must be made to the strain CH4-1 that, when grown on glucose is, by far, the best enzyme producer.

Only the strain CH5-4 presented a better enzyme production yield when grown on lactose, although this value is lower than the best-performing strains growing in lactose.

For other strains (CCT 3172, CH2-2, CH0-1, CH1-1, and CH5-3) the endo-PG activity was similar regardless of the carbon source – glucose or lactose.

In all cases enzyme productivity is between two- and three-fold lower when lactose was used, due to the very low substrate consumption rate.

Characterisation of flocculation ability of tested yeast strains

During the fermentation, biomass samples were taken and observed under a microscope (Fig. 3) since no flocs were visible in the fermentation flask by direct observation of the culture.

The sugar used as carbon source had an important role on floc formation - with glucose as carbon source the cells remained powdery. However, when lactose was used, all strains formed small flocs containing around 60 cells per floc (Fig. 3). Such a result was not expected, since glucose and lactose are reported to have no effect on floc formation (*13*). A possible explanation may be related to the fact that growth on lactose was very slow and cells might have aggregated not because of the carbon source but in response to stress conditions. The reduced micromixing resulting from the lower biomass activity for growth on lactose may also contribute to cell aggregation.

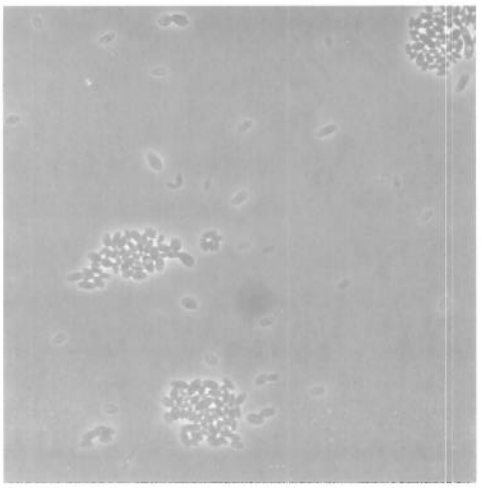

Fig. 3. Flocs of strain CH5-3 using lactose as carbon source

Conclusions

This work allowed for the characterisation of the effect of carbon source – glucose or lactose – on endo-polygalacturonase production using *K. marxianus* strains isolated from cocoa fermentation. With lactose as carbon source, strain CH5-3 produced the greatest amount of enzyme while for glucose-grown cells the best producer was strain CH4-1. It was confirmed for all strains tested, that endo-PG production is growth related, regardless of the carbon source, confirming the results obtained by Schwan and Rose (*12*) for the strain *K. marxianus* CCT 3172, and Wimborne and Rickard (*14*) with other strains of *Kluyveromyces marxianus*.

In all cases, carbon source plays a very important role in endo-PG production – enzyme productivity was significantly lower when yeasts were grown on lactose as a consequence of a correspondingly lower rate of substrate consumption. Since all the strains were originally isolated from non-lactose containing cocoa fermentations, further selection for improved growth on lactose may prove profitable.

With respect to flocculation, all strains could flocculate when grown on lactose but could not when grown on glucose. The presence of small yeast flocs when cells are grown in lactose makes it possible to consider the use of these strains for endo-PG production in high cell density bioreactors using flocculating yeasts, since flocculation can be improved in continuous systems, as described by Mota and Teixeira (*15*). However, the low enzyme productivity obtained when lactose is used as substrate, may hinder the utilization of these systems. Better endo-PG producing strains with flocculation ability are needed when grown on lactose.

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Izolacija, procjena i karakterizacija sojeva *Kluyvermyces* koji flokuliraju i proizvode pektinazu

Sažetak

Ispitano je 11 sojeva Kluyveromyces marxianus kako bi se odabrali najbolji proizvođači ekstracelularne endo-poligalakturonaze (endo-PG), koristeći glukozu i laktozu kao izvore ugljika. Ispitana je i sposobnost flokuliranja. Četiri od sedam sojeva proizvodili su više enzima ako se koristila glukoza kao supstrat. Samo je jedan soj bio bolji proizvođač endo-PG kada je laktoza bila izvor ugljika, iako je proizveo manje enzima nego najbolji soj što je rastao na laktozi. Najviše enzima dodavao je soj K. marxianus CH4-1 rastom na glukozi. U svim slučajevima, kao posljedica smanjene brzine utroška supstrata, proizvodnja enzima bila je dva do tri puta manja ako su kvasci bili uzgajani na laktozi. Isto se tako pokazalo da sojevi stvaraju flokule samo ako rastu na laktozi; na glukozi su stanice ostajale praškaste.