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PRODUCTION OF EXTRACELLULAR DEBRANCHING ACTIVITY BY AMYLOLYTIC YEASTS

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SUMMARY

The production of extracellular pullulan-degrading enzymes by several amylolytic yeast strains was studied. The highest activity was obtained with species of *Endomycopsis*, *Lipomyces*, *Filobasidium*, *Leucosporidium*, and *Trichosporon*, and also with some amylase-hyperproducing mutants. The most active strains are potentially valuable partners for intergeneric protoplast fusion.

INTRODUCTION

In recent years, considerable research has been devoted to starch-degrading yeasts. In addition to *Endomycopsis fibuligera* utilized in an industrial-scale process (Skogman, 1976), other species with interesting features for the production of single cell protein from starchy materials were found in the genus *Lipomyces* (Sà-Correia and van Uden, 1981) and *Schwanniomyces* (Oteng-Gyang *et al.*, 1980; Touzi *et al.*, 1982; Wilson *et al.*,1982). The potential application of *Schwanniomyces* species for ethanol production from starch has been proposed (Calleja *et al.*, 1982; Wilson *et al.*,1982; Frelot *et al.*, 1982; Moresi *et al.*, 1983). Economical utilization of these and other amylolytic species probably is not feasible due to their limited ethanol tolerance (Wilson *et al.*, 1982; De Mot *et al.*, 1984a). This problem could be circumvented by the construction of an active amylolytic *Saccharomyces* strain with excellent fermentation characteristics (Stewart *et al.*, 1982; Spencer and Spencer, 1983; Tubb, 1983).

The most active yeast species produce both α-amylase and

and glucoamylase(s) (Verachtert and De Mot, 1984). In the literature, limited information is available on the occurence of extracellular debranching enzymes from yeasts (Sukhumavasi *et al.*, 1975; Sills and Stewart, 1982; Wilson and Ingledew, 1982; Spencer-Martins, 1982; Stewart *et al.*, 1982). Hydrolysis of the 1,6-linkages in starch by debranching enzymes is essential for a quick and efficient conversion (Marshall, 1982; Gerghmans and Norman, 1982; Norman, 1982). Pullulanase and isoamylase are specific debranching enzymes, but several glucoamylases also possess a debranching side-activity (Fogarty and Kelly, 1980).

In this paper we report on the secretion of debranching activity by several starch-degrading yeasts, including some species recently characterized in our laboratory (De Mot *et al.*, 1984b).

MATERIALS AND METHODS

MICROORGANISMS. The yeast strains used with their origin and code number are listed in table 1. Stock cultures were maintained at 6 °C on slants of the following medium : soluble starch (Difco Laboratories, Detroit, USA) 1% (w/v), yeast nitrogen base (Difco) 0.67% (w/v), agar 2% (w/v),buffered at pH 5.5 with 75 mM sodium diphosphate-tartaric acid.

CULTURE CONDITIONS. The yeasts were grown at 29 °C on a reciprocal shaker in 150 ml Erlenmeyer flasks containing 30 ml of buffered yeast nitrogen base medium supplemented with 0.6% (w/v) soluble starch (E. Merck, Darmstadt, FRG)(De Mot *et al.*, 1984c). A lower incubation temperature (20 °C) was applied for *Trichosporon pullulans*. At 24 h intervals, small samples were taken and the cells removed by centrifugation at 2 °C (8,000 g; 15 min). The supernatant was assayed for total amylolytic activity and pullulanase activity.

ENZYME ASSAYS. Total amylolytic activity was determined by the dinitrosalicylate method as described previously (De Mot *et al.*, 1984c), except that soluble starch (Merck) 2% (w/v) was substituted for Zulkowsky soluble starch.

Pullulanase activity was determined by measuring the amount of reducing sugars liberated from pullulan following incubation of 0.2 ml enzyme solution with 0.5 ml of substrate solution at 40 °C for 45 min. The substrate solution contained 1% (w/v) pullulan (Sigma Chemical Company, St. Louis, USA) in 50 mM McIlvaine buffer pH 5.4. Reducing sugars were measured with the dinitrosalicylate reagent using a maltotriose (Sigma) standard solution (5 mM). One enzyme unit (U) was defined as the amount of reducing sugars (micromoles, expressed as maltotriose equivalents) produced in 45 min under the specified conditions.

RESULTS AND DISCUSSION

The yeast strains used in this study were selected for their capability of hydrolysing starch quickly and extensively (De Mot *et al.*, 1984b, 1984c). The highest values of extracellular pullulanase activities measured during growth in the medium with soluble starch are listed in table 1. An estimation of the contribution of pullulanase-like activity to the total amylolytic activity was obtained by the ratio of pullulanase activity over total amylolytic activity.

The extracellular pullulanase-like activity was lower than 0.5 U/ml for most species of *Candida*, *Cryptococcus*, *Pichia*, *Schwanniomyces* and *Torulopsis*. Secretion of pullulanase activity by *Schwanniomyces* species and *P. kurtonii* was reported earlier by Sills and Stewart (1982). *E. fibuligera* IGC 3961, *F. capsuligenum*, *L. capsuligenum*, *Tr. pullulans* and especially *Lip. kononenkoae* IGC 4052 produced considerably higher debranching activities. These yeasts are also characterized by a higher pullulanase activity relative to the total amylolytic activity. The strongest depolymerisation of pullulan was obtained with the hyperproducing mutant strains of *Lip. kononenkoae* IGC 4052 and of *Sch. castellii* ATCC 26077 (R69, R91). Strain R91 gave the highest relative amount of pullulanase activity.

A number of yeast strains degrading soluble starch only partially and lacking α-amylase (De Mot *et al.*, 1984b, 1984c), produced only low amounts of extracellular pullulanase activity (0-0.2 U/ml). This was the case for *Botryoascus synnaedendrus*, *C. ishiwadae*, *C. naeodendra*, *C. viswanathii*, *P. stipitis*, *P. vini*, *Saccharomycopsis malanga*, *T. molischiana* and *Tr. cutaneum* (data not listed).

The extracellular enzymes involved in the hydrolysis of pullulan by these yeast species, probably are not specific debranching enzymes. Extracellular isoamylase is produced by *Lip. kononenkoae* (Spencer-Martins, 1982), but the enzyme is not active on pullulan. The secretion of isoamylase or pullulanase by other yeast species has not yet been reported. We previously showed that α-amylase was produced by all the species from table 1 (De Mot *et al.*, 1984b, 1984c). Pu-

TABLE 1. PRODUCTION OF EXTRACELLULAR PULLULANASE BY STARCH-DEGRADING YEASTS

Generic name[7]	Number	Origin[1]	Pullulanase (U/ml)	Ratio[2]
C. homilentoma	CBS 6312	a	0.2	0.03
	CBS 6099	a	0.1	0.02
C. silvanorum	CBS 6274	a	0.1	0.02
C. tsukubaensis	CBS 6389	a	0.4	0.03
Cr. flavus	UCD-FS&T 68184	b	0.2	0.04
E. capsularis	CCY 42-5-3	c	0.4	0.02
E. libuligera	LCC 240	d	0.2	0.03
	IGC 3954	e	0.1	0.01
	IGC 3961	e	0.7	0.06
	IGC 3961-18[3]	f	0.5	0.04
F. capsuligenum	CCY 71-1-1	c	1.1	0.09
	CBS 4381	a	0.6	0.06
L. capsuligenum	CCY 64-2-4	c	0.5	0.04
	CCY 64-5-1	c	1.2	0.10
Lip. kononenkoae	IGC 4051	e	0.8	0.09
	IGC 4052	e	3.2	0.08
	IGC 4052-I[4]	f	3.4	0.06
	IGC 4052-II[4]	f	3.6	0.06
P. kurtonii	CBS 6141	g	0.4	0.17
Sch. alluvius	IGC 2829	e	0.1	0.02
	CCY 47-2-3	c	0.1	-
	CCY 47-2-8	c	0.3	0.07
	SA-2D6[5]	h	0.3	0.06
Sch. castellii	CBS 2863	g	0.3	0.05
	CCY 47-3-3	c	0.3	0.04
	CCY 47-3-6	c	0.3	0.06
	ATCC 26077	h	0.3	0.05
	R69[6]	h	1.3	0.10
	R91[6]	h	3.2	0.25
Sch. occidentalis	CBS 1153	g	0.3	0.07
	CBS 819	a	0.2	0.04
	CBS 2864	a	0.7	0.08
	LCC 1401	d	0.2	0.03
	CCY 47-1-1	c	0.2	0.04
	CCY 47-1-4	c	0.2	0.04
	CCY 47-1-7	c	0.2	0.05
	CCY 47-1-12	c	0.1	0.02
T. ingeniosa	CCY 26-22-1	c	0.1	-
Tr. pullulans	IGC 3488	e	1.2	0.15

(1) a = CBS, Baarn, The Netherlands; b = F. Federici, University of Perugia, Perugia, Italy; c = J. Augustin, Slovak Academy of Sciences, Bratislava, Czechoslovakia; d = I. Russell, Labatt Brewing Company, London, Canada; e = N. van Uden, Instituto Gulbenkian de Ciência, Oeiras Portugal; f = C. Cabeça-Silva, idem; g = P. Galzy, Chaire de Génétique et de Microbiologie, Montpellier, France; h = W. Ingledew, University of Saskatchewan, Saskatoon, Canada

(2) Ratio of pullulanase over total amylase activity

(3) Mutant strain from Cabeça-Silva (1982)

(4) Mutant strains obtained by van Uden *et al.* (1980)

(5) Mutant isolated by Wilson (1981)

(6) Mutants described by Dhawale and Ingledew (1983)

(7) *C=Candida, Cr=Cryptococcus, E=Endomycopsis, F=Filobasidium, L= Leucosporidium, Lip=Lipomyces, P=Pichia, Sch= Schwanniomyces, T=Torulopsis, Tr=Trichosporon*

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rified yeast α-amylases cannot hydrolyse pullulan (Wilson and Ingledew, 1982; Stewart *et al.*, 1982; Verachtert *et al.*, 1984), but substantial amounts of glucose can be liberated from pullulan by some yeast glucoamylases (Sukhumavasi *et al.*, 1975; Wilson and Ingledew, 1982; Stewart *et al.*, 1982; Verachtert *et al.*, 1984). Thus it may be possible that the extracellular glucoamylases are responsible for the degradation of pullulan by the yeasts studied, and that the enhanced debranching activity observed with several mutant strains can be attributed to an increased glucoamylase production.

Protoplast fusion is a valuable technique for the improvement of industrial yeast strains (Spencer and Spencer, 1983) and has been used to introduce the dextrin degradation characteristic of *Saccharomyces diastaticus* into *S. uvarum* and *S. cerevisiae* (de Figueroa *et al.*, 1984). However, *S. diastaticus* is rather unattractive as a fusion partner as no α-amylase and debranching activity are produced (Sills and Stewart, 1982). Our study shows that quite valuable candidates for intergeneric fusion are found in the genera *Filobasidium, Leucosporidium, Lipomyces* and *Trichosporon*. The utilization of appropriate hyperproducing mutant strains offers an attractive alternative. Stable intergeneric protoplast fusion products of *S. uvarum* and a derepressed *Schwanniomyces* mutant were reported recently (Van de Spiegele and Iserentant, 1984).

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