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Pullulan production by color variant strains of *Aureobasidium pullulans*

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SUMMARY

Naturally occurring 'color variant' strains of *Aureobasidium pullulans* are distinguished from typical strains by their brilliant pigmentation, overproduction of secreted enzymes (xylanase), and low DNA relatedness. Color variants have not previously been examined for pullulan secretion. Among five independently isolated color variants, strains NRRL Y-12,974 and YB-4026 made the greatest amounts of pullulan from cornstarch, with conversion efficiencies of about 10%. Neither color variant nor typical strains made significant amounts of pullulan from the unconventional lactose or xylan substrates. Pullulan yields were inversely correlated with biomass production. Pullulan production thus appears to be a variable characteristic of both color variant and typically pigmented strains of *A. pullulans*, regulated by specific inducers during growth limitation.

INTRODUCTION

Pullulan is an extracellular polysaccharide from the yeast-like fungus *Aureobasidium pullulans* (synonym *Pullularia pullulans*) (for reviews, see Refs. 9, 14, 24, 30). The primary structure of pullulan is a linear chain of maltotriose subunits in α-(1,6) linkage. The α-(1,6) bonds interrupt the regularity of what would otherwise be an amylose chain, resulting in enhanced solubility and structural flexibility. Consequently, pullulan possesses distinctive film- and fiber-forming characteristics not found in amylose. Pullulan can be formed into compression moldings that resemble polystyrene, or into fibers that resemble nylon [28]. Water-soluble films can be formed that are impermeable to oxygen; partial or complete water insolubility may be obtained by controlled esterification or etherification [28].

Naturally occurring 'color variant' strains of *A. pullulans* were first described in 1975 [27]. These

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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tropical isolates exhibited brilliant pigments of red, yellow, pink or purple. Typically pigmented strains were off-white to black in appearance (due to melanin). Color variants were classified as *A. pullulans* because of their microscopic appearance and carbon assimilation patterns. Nevertheless, preliminary comparisons of nuclear DNA complementarity now suggest that color variants are not highly related to typically pigmented strains of *A. pullulans*.

Recently, we discovered that color variants secrete the enzyme xylanase (EC 3.2.1.8) in remarkable yields [20]. Xylanase from color variants had properties similar to enzyme from typically pigmented strains, but was overproduced at levels two orders of magnitude greater than those from typical strains [19].

Pullulan is produced by only certain strains of *A. pullulans*, and by no other known species. We wished to test the capacity of color variants to produce pollulan, as a taxonomic characteristic of this group of strains. Since color variants secreted the enzyme xylanase in high yields, we hoped that polysaccharide secretion would also be elevated in these strains. The physiological regulation of pullulan synthesis by color variants was also tested. In previous studies (of typically pigmented strains) pullulan production was induced by glucose or fructose [6,8], and highest yields were obtained from starch-grown cultures [28,29]. Lactose and xylose were not inducers [13,21]. In addition to sucrose and starch, the unconventional lactose and xylan substrates were therefore tested. The resulting survey of *A. pullulans* strains for pullulan production is to our knowledge the largest of its kind to be published, and provides the first side-by-side comparison of several frequently used research strains.

MATERIALS AND METHODS

Organisms

A. pullulans strains may be divided into three groups (Table 1). Five independently isolated, naturally occurring color variant strains were previously described: NRRL Y-2311-1, Y-6754a, Y-12,974, YB-4026, and YB.4588 [19,20,27]. Four typically pigmented strains, never before examined for pullulan production, included NRRL Y-2312, Y-6992, YB-4029 and YB-4587. Strain YB-4029 was a coisolate of color variant YB-4026; YB-4587 was a coisolate of color variant YB-4588 [27]. Nine additional strains previously described as pullulan producers served as positive controls. Strain Y-2567, synonymous with QM-3092, has been used by Catley and others in numerous studies of pullulan production. Strain Y-17,005 has been frequently employed in pullulan studies by Ueda and colleagues, as strain S-1. Seven other strains are cited in publications or patents concerning pullulan, as detailed in Table 1.

Strain NRRL Y-12,996 was received from S.C. Jong, American Type Culture Collection, Rockville, MD; strain Y-12,999 was from J.E. Zajic, Petroleum Bio-Resources, El Paso, TX; strains Y-12,997, Y-12,998, Y-17,000 and Y-17,001 were from I. Banno, Institute for Fermentation, Osaka, Japan; strain Y-17,005 was from S. Ueda, Fukuoka, Japan. Other strains were from the ARS Culture Collection, Peoria, IL.

Growth medium and cultivation

Basal medium was similar to several previously used in pullulan studies [2,10,23,29], and contained per liter of deionized water: 2.0 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 1.0 g KH_2PO_4, and 0.4 g yeast extract (Difco). Carbon source was 2.0% (w/v) sucrose, cornstarch, lactose, or xylan. Cornstarch and xylan formed stable suspensions at 2.0%, but were not easily prepared at higher concentrations.

Media were used at an unadjusted pH of 5.2. In preliminary experiments, culture pH rapidly returned during growth to pH 5.0–6.0, from initially adjusted values of either pH 2.5 or pH 7.0. In contrast, previous reports described a drop in culture pH during pullulan fermentation, to the detriment of pullulan yields [7,18]. Higher carbon concentrations used in those studies may have resulted in a significant accumulation of organic acids.

Cultures were 10 ml, in Morton-closured 50-ml

Table 1

Strains of *A. pullulans* used in this study

NRRL strain No.	Type[a]	Equivalent numbers, apparent synonyms	Ref.
Y-2311-1	CV	(Light pink, derivative of red Y-2311)	19,20,27
Y-6754a	CV	(purple)	19,20,27
Y-12,974	CV	(pink)	19
YB-4026	CV	(pink)	19,20,27
YB-4588	CV	(red)	19,20,27
Y-2312	TP	YB-2461	19,20
Y-6992	TP	IGC 4209	19,20
YB-4029	TP		19,20,27
YB-4587	TP		19,20,27
Y-2567	PP	QM-3092, QM-3090, ATCC 9348, F-44	6–8,19,20,23
Y-6220	PP	ATCC 34647, M-42	19,20
Y-12,996	PP	ATC 42023	16
Y-12,997	PP	*Dematium pullulans*, IFO 4464	16
Y-12,998	PP	IFO 6353	26
Y-12,999	PP	PBR-Pp-Km-3	25
Y-17,000	PP	*Pullularia fermentans*, IFO 6401	16
Y-17,001	PP	*P. fermentans*, IFO 6402	16
Y-17,005	PP	S-1	25,26

[a] Strain types: CV, color variant strains of *A. pullulans*; TP, typically pigmented strains of *A. pullulans*, previously uncharacterized with respect to pullulan production; PP, pullulan producer, strains of *A. pullulans* described in published literature as sources of pullulan.

Erlenmeyer flasks, shaken at 200 rpm at 28°C for 9 days (New Brunswick model G-53 shaker, stroke ca. 2 inches). Extended culture periods were used because pullulan yields rather than molecular weights were of interest; pullulan molecular weight may be reduced late in stationary growth phase [5,11].

Pullulan isolation and assays

Culture supernatants were cleared of cells by centrifugation (30 min at 15 000 rpm, Sorvall SS-34 rotor). Pullulan was precipitated with an equal volume of tetrahydrofuran (THF) followed by similar centrifugation. THF was chosen for its reported efficiency and specificity towards pullulan [15]. THF precipitates and biomass were quantitated as dry weights from samples dried to constant weight, for over 3 h at 80°C under vacuum.

Authentic pullulan was estimated by sensitivity to pullulanase. Assays were performed with air-dried THF precipitates, since thoroughly dehydrated pullulan resolubilized poorly, as has been previously reported [3]. Precipitated pullulan was resuspended at 0.1% (w/v) in 50 mM sodium acetate, pH 5.0. Pullulanase (from *Enterobacter aerogenes*) was added to 0.1 U/ml and incubated for over 15 h at 25°C. Maltotriose reducing sugar equivalents were revealed by the dinitrosalicylic acid method [22]. Commercial pullulan was over 95% sensitive to pullulanase by these methods.

All dry weight and pullulanase values were means of two to four culture replicates; standard deviations are given.

Melanin was qualitatively determined by visual inspection of culture broths and precipitates.

DNA relatedness determinations

Methods for the isolation and purification of total nuclear DNA were previously described [17]. The extent of nuclear DNA complementarity was

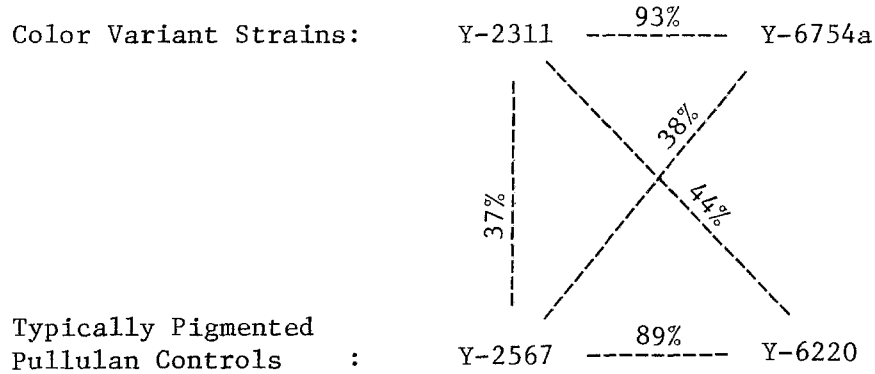

Fig. 1. Extent of DNA relatedness between representative color variant and typically pigmented pullulan control strains. DNA hybridizations were performed as described in Materials and Methods. NRRL YB-4619 is *Sterigmatomyces halophilus*, taxonomically unrelated to *A. pullulans*, and served as a negative control. Standard deviations were less than 5% of means.

determined spectrophotometrically [17]. *A. pullulans* strains compared for complementarity were found to have identical guanine plus cytosine contents of 51.0% (within a single standard deviation).

Chemicals

Cornstarch, xylan, maltotriose, pullulan and pullulanase were from Sigma Chemical Co., St. Louis, MO. Sucrose, lactose, and yeast extract were

Table 2

Biomass yields and carbon source utilization

Strains	Type[a]	Biomass dry weights (g/l, ± S.D.)			
		sucrose	starch	lactose	xylan
Y-2311-1	CV	3.9 ± 0.8	5.3 ± <0.1	1.3 ± 0.1	2.5 ± 0.1
Y-6754a	CV	5.9 ± <0.1	4.8 ± 0.2	1.3 ± <0.1	3.0 ± 0.7
Y-12,974	CV	7.3 ± <0.1	5.4 ± 0.5	1.5 ± <0.1	3.0 ± 0.2
YB-4026	CV	6.1 ± 0.1	4.2 ± 0.2	0.8 ± 0.1	2.6 ± 0.3
YB-4588	CV	5.4 ± 0.2	5.7 ± 0.4	1.0 ± 0.2	1.2 ± 0.3
Y-2312	TP	5.7 ± 0.7	6.6 ± 0.1	1.4 ± 0.1	2.5 ± 1.1
Y-6992	TP	5.4 ± 0.1	4.9 ± <0.1	0.4 ± 0.1	2.5 ± 0.2
YB-4029	TP	6.1 ± 0.1	4.2 ± 0.1	0.9 ± <0.1	3.5 ± 0.9
YB-4587	TP	4.5 ± 0.4	3.0 ± <0.1	1.4 ± <0.1	1.7 ± 0.3
Y-2567	PP	5.7 ± 0.1	5.6 ± 0.2	3.2 ± 0.2	1.8 ± 0.8
Y-6220	PP	4.8 ± 0.2	3.2 ± 0.2	1.4 ± 0.1	1.3 ± 1.0
Y-12,996	PP	4.5 ± 0.4	2.4 ± 0.5	1.3 ± <0.1	2.6 ± 0.3
Y-12,997	PP	5.3 ± 0.3	3.7 ± 0.1	3.4 ± 1.1	2.5 ± 0.6
Y-12,998	PP	7.8 ± 0.8	6.6 ± 0.1	2.6 ± 1.2	2.3 ± 0.1
Y-12,999	PP	4.7 ± 0.5	3.6 ± 0.6	1.4 ± 0.2	4.5 ± 1.1
Y-17,000	PP	6.4 ± 0.4	7.3 ± 0.2	1.0 ± 0.1	3.2 ± 0.3
Y-17,001	PP	8.4 ± 0.6	5.0 ± <0.1	1.4 ± 0.2	4.1 ± 0.6
Y-17,005	PP	4.4 ± 0.5	3.9 ± 0.5	1.6 ± 0.1	3.2 ± 0.7

[a] Strain types: CV, color variant strains of *A. pullulans*; TP, typically pigmented strains of *A. pullulans*, previously uncharacterized with respect to pullulan production; PP, pullulan producer, strains of *A. pullulans* described in published literature as sources of pullulan.

from Difco Laboratories, Detroit, MI. Dinitrosalicylic acid was from Aldrich Chemical Co., Milwaukee, WI. Other chemicals were reagent grade.

RESULTS AND DISCUSSION

DNA relatedness of color variant and typically pigmented strains of A. pullulans

Fig. 1 illustrates the extent of DNA relatedness among representative color variant and typically pigmented strains of *A. pullulans*. Independently isolated color variant strains Y-2311 and Y-6754a showed essentially complete homology. Typically pigmented strains Y-2567 and Y-6220 were equally similar. However, DNA relatedness between color variant and typical strains ranged from 37% to 44%. These preliminary values suggest that color variants are a genetically distinct population that may represent a new species recently diverged from typical strains. This question will be dealt with more extensively in a future publication.

Biomass yields and carbon source utilization

As shown in Table 2, all 18 strains of *A. pullulans* grew well on sucrose, producing from 3.9 to 8.4 g of dry weight biomass per liter. Yields on cornstarch, while substantial for all strains, averaged 17% lower than those from sucrose-grown cultures. Growth yield limitation of starch cultures may be relevant to polysaccharide yields, as discussed below.

Strains grew poorly on lactose with the exception of Y-2567, Y-12,997, and Y-12,998. Although these three strains were previously described as pullulan producers, pullulan has never been reported in significant yields from lactose-grown cultures [13,21]. Growth yields on xylan varied considerably, with no apparent relationship to strain type.

Table 3

Total extracellular polysaccharide yields

Strains	Type[a]	THF precipitates (g/l ± S.D.)			
		sucrose	starch	lactose	xylan
Y-2311-1	CV	1.0 ± <0.1	0.0 ± <0.1	0.4 ± <0.1	1.9 ± 0.1
Y-6754a	CV	1.9 ± 0.2	2.3 ± 0.1	0.5 ± 0.3	1.8 ± 0.2
Y-12,974	CV	4.5 ± 0.4[b]	6.4 ± 0.4[b]	0.3 ± 0.1	1.9 ± 0.5
YB-4026	CV	1.9 ± 0.3	6.3 ± 0.1[b]	0.3 ± 0.3	1.9 ± 0.5
YB-4588	CV	1.8 ± <0.1	0.4 ± 0.2	0.3 ± 0.3	1.9 ± 0.1
Y-2312	TP	3.3 ± 0.1	0.5 ± 0.3	1.0 ± <0.1	1.4 ± 0.2
Y-6992	TP	2.4 ± <0.1	0.3 ± 0.3	0.1 ± 0.1	1.6 ± <0.1
YB-4029	TP	2.2 ± 0.4	3.7 ± 0.3	0.8 ± <0.1	2.1 ± 0.1
YB-4587	TP	1.2 ± <0.1	7.7 ± 0.9[b]	0.4 ± 0.2	1.8 ± <0.1
Y-2567	PP	2.6 ± 0.2	4.5 ± 0.1[b]	0.7 ± 0.1	1.9 ± 0.5
Y-6220	PP	3.1 ± 0.3	9.6 ± 1.2[b]	0.6 ± 0.4	2.1 ± 0.3
Y-12,996	PP	2.9 ± 0.3	10.2 ± 1.0[b]	0.7 ± 0.3	2.1 ± 0.1
Y-12,997	PP	2.6 ± <0.1	8.1 ± 0.5[b]	0.5 ± 0.3	1.9 ± 0.5
Y-12,998	PP	5.2 ± 0.2[b]	1.7 ± 0.3	0.6 ± 0.4	2.4 ± 0.4
Y-12,999	PP	2.7 ± 0.1	9.5 ± 1.5[b]	0.7 ± 0.1	2.4 ± <0.1
Y-17,000	PP	0.3 ± 0.3	0.0 ± <0.1	0.1 ± 0.1	1.7 ± 0.5
Y-17,001	PP	2.6 ± 0.2	1.1 ± 0.1	0.6 ± 0.2	2.3 ± 0.1
Y-17,005	PP	5.6 ± 0.6[b]	4.0 ± 0.2	0.8 ± <0.1	2.1 ± 0.1

[a] Strain types: CV, color variant strains of *A. pullulans*; TP, typically pigmented strains of *A. pullulans*, previously uncharacterized with respect to pullulan production; PP, pullulan producer, strains of *A. pullulans* described in published literature as sources of pullulan.

[b] Polysaccharide yield exceeds 20% substrate conversion.

Total extracellular polysaccharide yields

Table 3 summarizes dry weights of THF-precipitated material from test culture supernatants; these yields are operationally defined as total extracellular polysaccharide. Among strains previously described as pullulan producers (strain type 'PP'), seven of nine converted over 20% of substrate to polysaccharide under our growth conditions. These highest yields were all from the conventional substrates sucrose or starch. Strain Y-12,996 converted over half of initial starch, to a mean of 10.2 g/l product. Strains Y-6220, Y-12,997, and Y-12,999 had yields representing over 40% conversion of starch. Strains Y-12,998 and Y-17,005 were exceptional in that significant polysaccharide yields were obtained from sucrose- but not from starch-grown cultures. As discussed below, the product from these particular sucrose cultures does not appear to be pullulan.

Among the nine strains previously uncharacterized with respect to pullulan production (five color variant and four typically pigmented), only three exceeded 20% conversion of substrates. Color variant strains Y-12,974 and YB-4026 effected approximately 30% conversion of cornstarch; Y-12,974 was also productive on sucrose. Starch-grown cultures of typical strain YB-4587 produced polysaccharide in yields similar to those from the two color variants. Interestingly, YB-4587 was a coisolate of color variant YB-4588; typical strain YB-4029 was a coisolate of productive color variant YB-4026. The capacity for polysaccharide production was thus correlated neither with strain type nor with particular isolations.

Polysaccharide yields from lactose cultures were below 5.0% of substrate for all strains, including the three that showed reasonable growth on lactose. Polysaccharide yields from xylan cultures were only slightly higher at 12% or less, with little strain variability.

Pullulan content of extracellular polysaccharides

Although some authors broadly define 'pullulan' as all polysaccharides secreted by *A. pullulans,* we

Table 4

Pullulan content of extracellular polysaccharides

Strain	Type[a]	Substrate	Pullulan		
			% total polysaccharide	% conversion efficiency	melanin[b]
Y-12,974	CV	Sucrose	25 ± 2	6	−
Y-12,974	CV	Starch	35 ± 1	11	−
YB-4026	CV	Starch	29 ± 2	9	−
YB-4587	TP	Starch	33 ± 1	13	−
Y-2567	PP	Starch	57 ± 2	13	−
Y-6220	PP	Starch	54 ± <1	26	−
Y-12,996	PP	Starch	45 ± 3	23	+
Y-12,997	PP	Starch	51 ± 1	21	−
Y-12,998	PP	Sucrose	1 ± 2	1	+
Y-12,999	PP	Starch	44 ± 1	21	+
Y-17,005	PP	Sucrose	<1 ± <1	<1	+
Y-2312	TP	Lactose	7 ± 7	<1	−
Y-12,999	PP	Xylan	9 ± 6	1	−

[a] Strain types: CV, color variant strains of *A. pullulans*; TP, typically pigmented strains of *A. pullulans,* previously uncharacterized with respect to pullulan production; PP; pullulan producer, strains of *A. pullulans* described in published literature as sources of pullulan.

[b] By inspection of cleared culture supernatants and THF precipitates.

were specifically interested in what we termed 'authentic' pullulan, i.e., α-(1,6)-linked polymaltotriose, particularly with regard to the taxonomic value of this trait for color variant strains. Previous studies have reported varying degrees of heterogeneity in pullulan-containing polysaccharide preparations [3,4,6]. Furthermore, yeast-like fungi other than *A. pullulans* have been reported to produce a variety of polysaccharides other than pullulan [14,24].

As an operational definition of authentic pullulan, pullulanase sensitivity was measured as described in Materials and Methods. Polysaccharides from cultures with yields over 4.0 g/l (20% conversion of substrate, Table 3) were tested. The results appear in Table 4.

Polysaccharide from starch-grown cultures of Y-2567, Y-6220, Y-12,996, Y-12,997, and Y-12,999 (strains previously identified as pullulan producers) were about 50% pullulan within a narrow range (44–57%). Conversion efficiencies of cornstarch to authentic pullulan were thus 13–26% for these strains. Strain Y-6220 produced the highest pullulan yields, whereas Y-2567 had the most pure product. As noted in Table 4, pullulan from Y-12,996 and Y-12,999 was contaminated with melanin.

As shown above, positive control strains Y-12,998 and Y-17,005 converted sucrose but not starch to polysaccharide at greater than 20% efficiency. Quite interestingly, polysaccharide from sucrose cultures of Y-12,998 and Y-17,005 was almost entirely resistant to pullulanase. Although melanin was observed in precipitates from these cultures (conceivably inhibitory to pullulanase or contributing to polysaccharide dry weight), melanin was also found in precipitates from two cultures containing over 40% measured pullulan. Since Y-12,998 and Y-17,005 have been used for a number of previous studies relating to pullulan (e.g., Refs. 8, 25, 26), it would be of considerable interest to determine the nature of these non-pullulan polysaccharides.

Color variant strains Y-12,974 and YB-4026 did produce authentic pullulan. Pullulan composition ranged from 25% to 35%, slightly lower than the purities from productive control strains. Typically pigmented strain YB-4587 similarly made a product of 33% pullulan. Starch conversion efficiencies (to authentic pullulan) by color variant strains Y-12,974 and YB-4026 thus averaged 10%, about half of the highest values from productive control strains, but similar to control strain Y-2567 and typical strain YB-4587. Polysaccharides from these color variant strains were not contaminated with melanin.

Although relatively low, the highest polysaccharide yields among lactose- and xylan-grown cultures were from strains Y-2312 and Y-12,999, respectively (Table 3). Precipitates from these cultures contained only low and variable amounts of authentic pullulan (Table 4).

In absolute terms, maximal pullulan yields reported here are about one-third of those of some reports, that claim up to 75% conversion of starch (e.g., Refs. 1, 28). Our culture conditions were not optimized for production; further, the relatively low carbon levels that we employed (2.0% w/v) may have reduced conversion efficiencies, since most pullulan production may occur after culture limitation (further discussed below). However, our results lead us to suspect that reported yields may suffer from problems of product impurity and moisture, often unexamined in simple dry weight determinations.

Biomass yields, culture limitation and pullulan production

As noted above, biomass yields from starch-grown cultures were lower on average than those from sucrose-grown cultures (Table 2). Growth limitation for nutrients other than carbon has been proposed to favor pullulan production [7,10,23]; in this regard, pullulan production would be similar to production of most other secondary metabolites. We examined the relationship between biomass and polysaccharide yields. Since cultures might be non-productive for a variety of reasons, only productive cultures were included (those from Table 4). Inverse correlations were indeed found between growth yields and both total polysaccharide and authentic pullulan yields (Fig. 2). Correlation coefficients (r values) for regressed lines were -0.83 for total po-

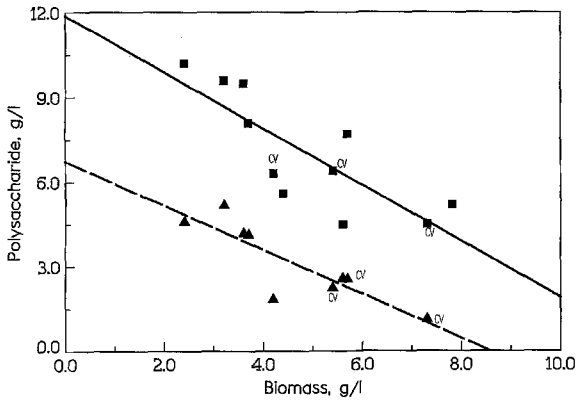

Fig. 2. Relationship between biomass yields and polysaccharide yields. Cultures effecting greater than 20% conversion of substrate to polysaccharide (those in Table 4) were included. Biomass yields were plotted against total polysaccharide yields (■) and against authentic pullulan (▲). Lines were fitted by linear regression. Points representing color variant strains are denoted 'CV'.

lysaccharide and −0.76 for authentic pullulan. Points representing color variants Y-12,974 and YB-4026 are identified on Fig. 2 (by 'CV'), and fit well into the trend. Regulation of pullulan production, by substrate induction and growth limitation, therefore appeared similar among all strains tested, including color variants.

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