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# AMYLOLYTIC ACTIVITY OF *SCHWANNIOMYCES OCCIDENTALIS* MUTANTS*

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Key words: *Schwanniomyces occidentalis*, amylolytic activity, α-amylase, glucoamylase.

UV irradiation and a multi-stage selection produced *S. occidentalis* strains with amylolytic activity increased from 17.5 to 65% in the case of α-amylase, and from 5.7 to 34% in the case of glucoamylase.

According to the literature, about 200 yeast are capable of utilizing starch as the only source of carbon and energy [1, 7]. They represent mainly species of the following genera: *Candida, Endomycopsis, Sporobolomyces, Torulopsis, Lipomyces, Schwanniomyces, Pichia,* and *Hansenula.* De Mot [3] added to the list of amylolytic strains also *Brettanomyces, Bullera, Cryptococcus, Filobasidum, Leucosporidium,* and *Trichosporon.*

*Schwanniomyces alluvius*, one of the 56 yeast strains studied by Spencer [11] metabolized starch with the yield of 38%.

It was reported in the literature [9, 14] that Schwanniomyces yeasts may be used in industral production of cellular protein, and in the production of ethanol from starch. The high degree of starch hydrolysis characterizing the Schwanniomyces strains is to be seen as due to α-and glucoamylase activities as well as to the so called "debranching" activities present in their amylolytic complex [10]. The activities of amylolytic enzymes may be increased by selections leading to the obtaining of strains immune to catabolic repression [13].

In this research we attempted to improve the *S. occidentalis* Y671 strain by mutations and selections of clones with increased amylolytic capabilities, with the view to using them to enrich starch media in protein content.

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## MATERIALS AND METHODS

### BIOLOGICAL MATERIAL

The experiments were carried out with S. occidentalis strain no. Y671, obtained from the All-Union Yeast Strains Collection in Moscow, and with mutants obtained by UV irradiation. The strains were stored in a routine manner on YPS agar slants (Table 1) and were reinoculated every 10 days. Cultures were maintained at 30°C for 48 h, and then stored at +4°C.

Table 1. Culture media

| Kind of medium (according to the literature) | | Purpose |
|---|---|---|
| YPG [2] (%) | | Selection of mutants; quantitative inoculations with biological material |
| yeast extract | 0.25 | |
| peptone tryptone | 0.5 | |
| glucose | 1.0 | |
| YPS [2] (%) | | Storage of strains |
| yeast extract | 0.25 | |
| peptone tryptone | 0.5 | |
| starch | 1.0 | |
| $M_0$ medium + starch (%) | | Selection of amylolytic mutants; studies of growth dynamics and amylolytic capabilities of yeasts |
| $(NH_4)_2SO_4$ | 0.3 | |
| $KH_2PO_4$ | 0.1 | |
| $MgSO_4 \cdot 7H_2O$ | 0.05 | |
| yeast extract | 0.05 | |
| starch | 1 to 5 | |
| pH = 5.0-5.5 | | |
| $M_0$ medium as above plus | | Selection of mutants; studies of amylolytic capabilities and resistance to catabolic repression |
| 2-deoxy-D-glucose | 0.1 | |
| agar | 2.0 | |
| Potato extract [2] (%) | | Estimation of mutants' growth and amylolytic activity |
| $(NH_4)_2SO_4$ | 0.5 | |
| $KH_2PO_4$ | 0.1 | |
| $CaCl_2$ | 0.3 | |
| pH = 5.0-5.5 | | |

### CULTURE MEDIA

The applied culture media listed in Table 1.

### YEAST CULTURE

The dynanics of mutants growth was assesses in shaker cultures (220-240 r.p.m., vibration amplitude 30 mm) on $M_0$ minimal medium, and on potato

extract. The culture was maintained at 30°C for 27 h. The medium was inoculated with a suspension of yeasts from the phase of exponential growth. The inoculum dose was 10% (V/V).

### MUTATION OF YEASTS

A cell suspension ($3.2 \times 10^6$ cell per $cm^3$) was applied to Petri dishes containing YPG, $M_0$ or $M_0$ + 2-DG media, which were then exposed to 30 or 60 seconds of UV irradiation. The source of UV rays was an L 8 type 380/550 W quartz lamp, placed 30 cm from the irradiated suspension.

### BIOMASS DETERMINATION

The culture yeast biomass was determined spectrophotometrically, and dry mass was expressed in $mg/cm^3$. Cell number (CFU) was determined using the plate method, YPG medium, with culture maintained at 30°C for 48 h. The number of colonies obtained was expressed as the number of colony forming units ($CFU/cm^3$).

### GROWTH PARAMETER DETERMINATION

Growth parameters were calculated from the following formulas [6]:

a) $$\mu = \frac{\ln x_t - \ln x_0}{t} \ (h^{-1})$$

where $\mu$ — specific growth rate ($h^{-1}$), $x_t$ — $CFU/cm^3$ at time t (end of the exponential growth phase), $x_0$ — $CFU/cm^3$ at time $t_0$ (beginning of exponential growth phase), and

b) $$T = \frac{\ln 2}{\mu} \ (h)$$

where T — generation time.

### ENZYMATIC ACTIVITY DETERMINATION

Alpha-amylase activity was determined by the Fischer-Stein method [5]. The assumed unit of activity was the amount of enzyme liberating 1 $\mu$mol of glucose in a 1% solution of soluble starch during 3 min. Glucoamylase activity was determined according to Spencer [12]. In this case the assumed unit of activity was the amount of enzyme liberating 1 $\mu$mol of glucose in a 1 % solution of soluble starch during 20 min in standard conditions (pH 4.5, 30°C).

### DETERMINATION OF REDUCING SUGARS

The content of reducing sugars in the culture medium was determined with the sodium salicylate reagent following the deproteinization and hydrolysis of the sample. Colour intensity was measured spectrophotometrically at $\lambda = 550$ nm wavelength. The results were read in mg glucose from the standard curve [8].

### STARCH DETERMINATION

The content of starch in the culture medium was determined colorimetrically according to Federici [4] using the iodine reagent. Extinction was measured at wavelength $\lambda = 610$ nm, and the reading was compared with a previously plotted standard curve. The relationship between starch concentration and extinction is represented by a straight line for starch contents ranging from 0.5 to 5.0 mg/cm$^3$.

## RESULTS AND DISCUSSION

### MUTATION OF S. OCCIDENTALIS Y671

The scheme of the mutation is shown in Fig. 1. The mutation-inducing factor were UV rays which, according to the data in Table 2, had a considerable lethal effect. Irradiation for 30 and 60 seconds reduced the cells' survival rate by

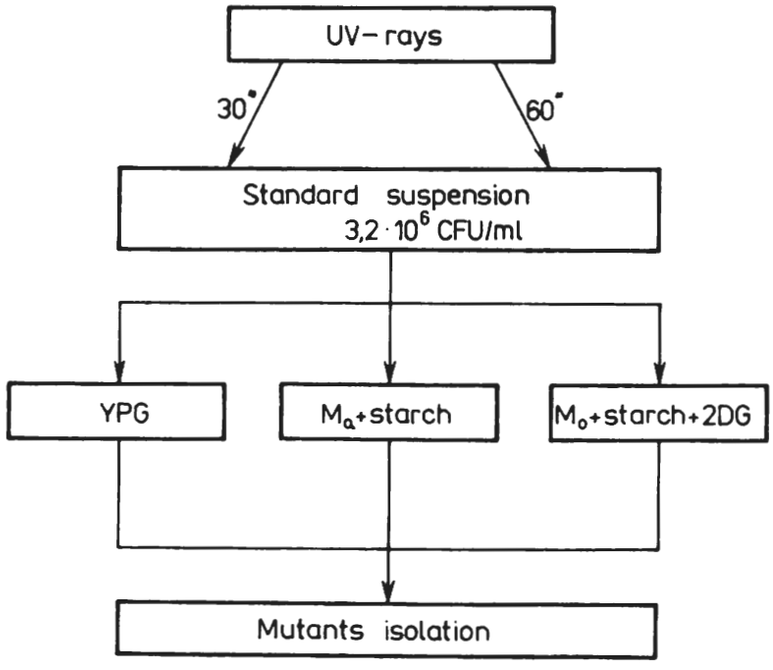


Fig. 1. Scheme of *S. occidentalis* mutation

$0.63 \times 10^{-3}$% to $1.62 \times 10^{-3}$%. The mutants of *S. occidentalis* Y671 were isolated from three media: YPG, $M_0$ + starch, and $M_0$ + starch + 2DG. A total of 21 mutants were isolated, and from this material the strains exhibiting

Table 2. Results of *S. occidentalis* Y671 mutation induced by UV irradiation

| Medium | Irradiation time (s) | Number of cells (CFU/cm$^3$) | | Survival rate (%) |
|---|---|---|---|---|
| | | initial | after mutation | |
| YPG | 30 | $3.2 \times 10^6$ | $5.2 \times 10^1$ | $1.62 \times 10^{-3}$ |
| | 60 | $3.2 \times 10^6$ | $3.1 \times 10^1$ | $0.97 \times 10^{-3}$ |
| YPG+2DG | 30 | $3.2 \times 10^6$ | $2.5 \times 10^1$ | $0.78 \times 10^{-3}$ |
| | 60 | $3.2 \times 10^6$ | $2.0 \times 10^1$ | $0.63 \times 10^{-3}$ |

increased amylolytic activity were selected. This was done by isolating strains producing the largest colonies (about 5 mm in diameter) or large zones of starch hydrolysis. The successive stages of mutants selection are described in Table 3.

The amylolytic mutants of *S. occidentalis* Y671 were selected in dish cultures on $M_0$ medium with starch. Their resistance to catabolic repression was checked in dish cultures on a starch medium with 2-deoxyglucose (Table 3). In the subsequent stages of selection the criteria for evaluating the obtained mutants were: colony dimensions, size of the starch hydrolysis zone, produced biomass level, and activity of the enzymatic complex (Table 3).

Table 3. Selection of amylolytic mutants

| Selection stages | Media | Assessment criteria |
|---|---|---|
| I. Isolation of mutants from selective media | $M_0$ + starch | colony dimensions, size of starch hydrolysis zone |
| II. Selection of strains resistant to catabolic repression | $M_0$ + starch + 2DG | colony dimensions, size of starch hydrolysis zone |
| III. Selection of mutants in shaker culture | $M_0$ + starch (1%) | biomass, starch utilization, activity of enzymatic complex |

ASSESSMENT OF THE MUTANTS' AMYLOLYTIC ACTIVITY

The relevent results, collected in Table 4, indicate that the mutants displayed various starch hydrolysis capabilities. The hydrolysis zones ranged from 16 to 22 mm. The ratio of starch hydrolysis zone diameter (R) to colony diameter (r), denoted q in the table, was in the range 3.2-4.4, and it was adopted as the criterion for selecting the mutants to the next stage of studies, namely to the assessment of activity in shaker cultures. These studies were performed with two mutants with the least ability to educe extra-cellular amylolytic enzymes (q = 3.2-3.4) and six mutants with q values of 4.0-4.4.

The results of detailed determinations in shaker cultures on $M_0$ medium are collected in Table 5. As can be seen, the mutants produced 5.7-6.0 g dry substance of biomass per dm$^3$, a yield amounting to 57-60% of the starch added to the medium. The amylolytic activity of the enzymes was found to vary. After 17 h of shaker culture, α-amylase activities ranged from 6.55 to 11.4 units/cm$^3$, and glucoamylase activities ranged from 34 to 51.6 units/cm$^3$.

Table 4. Screening of mutants in Petri dish cultures

| Strain | Diameter (mm) of | | q = R/r | Strains selected for further studies |
|---|---|---|---|---|
| | colony (r) | hydrolysis zone (R) | | |
| Y671/ 1 | 5 | 21 | 4.2 | + |
| 2 | 5 | 20 | 4.0 | |
| 3 | 5 | 16 | 3.2 | + |
| 4 | 5 | 17 | 3.4 | |
| 5 | 5 | 21 | 4.2 | |
| 6 | 5 | 22 | 4.4 | + |
| 7 | 5 | 22 | 4.4 | + |
| 8 | 5 | 21 | 4.2 | |
| 9 | 5 | 20 | 4.0 | + |
| 10 | 5 | 19 | 3.8 | |
| 11 | 5 | 20 | 4.0 | + |
| 12 | 5 | 20 | 4.0 | |
| Y671/DG/1 | 5 | 17 | 3.4 | + |
| 2 | 5 | 20 | 4.0 | |
| 3 | 5 | 20 | 4.0 | |
| 4 | 5 | 18 | 3.6 | |
| 5 | 5 | 22 | 4.4 | + |
| 6 | 5 | 21 | 4.2 | |
| 7 | 5 | 21 | 4.2 | |
| 8 | 5 | 22 | 4.4 | + |
| 9 | 5 | 19 | 3.8 | |

Table 5. Characteristic of *S. occidentalis* mutants in $M_0$ mediuum with 1% starch ($\tau$ = 17 h, t = 30°C)

| Strain \ Estimation | Dry mass ($g/dm^3$) | α-amylase ($U/cm^3$) | glucoamylase ($U/cm^3$) | Δα-amylase (%) | Δ glucoamylase (%) |
|---|---|---|---|---|---|
| Y671/3 | 5.7 | 6.55 | 34.0 | -5.80 | -11.9 |
| Y671/6 | 6.0 | 11.40 | 51.6 | 65.5 | 33.7 |
| Y671/7 | 5.7 | 10.0 | 46.0 | 44.7 | 19.1 |
| Y671/9 | 6.0 | 9.50 | 47.6 | 37.5 | 23.3 |
| Y671/12 | 6.0 | 8.25 | 50.0 | 19.5 | 29.5 |
| Y671/DG/1 | 5.7 | 9.20 | 46.8 | 33.3 | 21.2 |
| Y671/DG/5 | 5.7 | 8.10 | 40.8 | 17.2 | 5.70 |
| Y671/DG/8 | 5.7 | 10.5 | 51.6 | 52.2 | 33.7 |
| Y671 (parent strain) | 5.6 | 6.91 | 38.6 | 0 | 0 |

The mutants resistant to 2-DG displayed similar activities of amylolytic enzymes. The activity of extracellular α-amylase was 8.1-10.5 units/$cm^3$, and of glucoamylase 40.8-51.6 units/$cm^3$.

In seven of the studied mutants the amylolytic activity was found to be higher than in the parent strain. Alpha-amylase activity increased from 17.2 to 65.5%,

and glucoamylase activity from 5.7 to 33.7%. The only mutant to have an activity lower than that of the parent strain was Y671/3, which also produced the smallest starch hydrolysis zone in the preliminary tests (Tables 4 and 5).

The growths of the most active mutant Y671/6 and of the parent strain Y671 in $M_0$ medium with various concentrations of starch (1-5%) are compared in

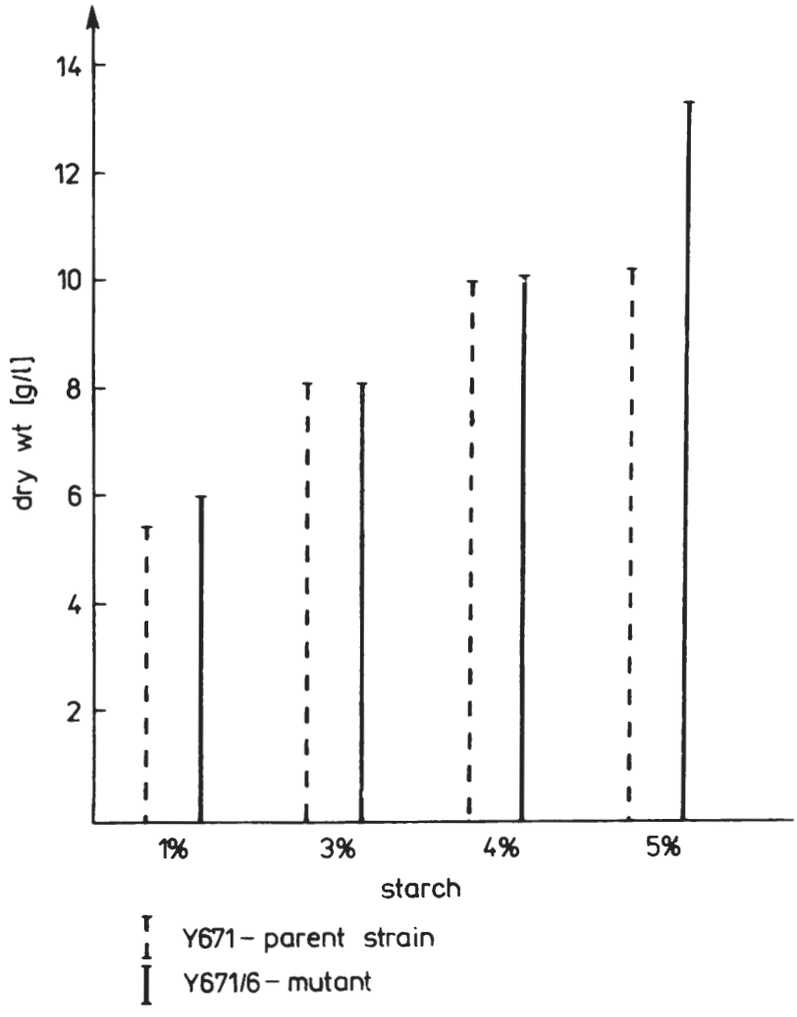


Fig. 2. Biomass production in $M_0$ media with different starch concentrations

Fig. 2. The strains produced 8.4-13.6 g biomass per $dm^3$, with the biomass yield coefficient ranging from 0.23 to 0.27. The mutant was clearly more productive at the high starch concentration of 5%. Starch hydrolysis by the strains was very intense, and by the 12th hour of culture starch was no longer detectable in the reaction with iodine solution, even when its concentration in the $M_0$ medium was 5% (Table 6).

The amylolytic capabilities of the most active mutants were also assessed in the potato medium (Table 7). The results indicate that in the investigated strains cell multiplication was high ($10^8/cm^3$), their growth parameters were good, and their amylolytic activity high. The α-amylase activity of 10.6-13.3 units/$cm^3$ was about four times higher than in the minimal medium $M_0$. Glucoamylase activity in the compared media was similar, and ranged from 59 to 67 units/$cm^3$ (Table 7).

The results concerning growth dynamics and amylolytic activity of selected mutants after six months storage in laboratory conditions are presented in Table 8 and Fig. 3. An assessment of the stability of biotechnological properties

Table 6. Dynamics of starch utilization by *S. occidentalis* strains in $M_0$ minimal medium

| Strain | Culture duration (h) | Starch concentration (mg/cm³) | | | |
|---|---|---|---|---|---|
| | | 10 | 30 | 40 | 50 |
| Y671 (parent strain) | 0 | 9.8 | 29.5 | 43.0 | 51.2 |
| | 4 | 1.0 | 15.2 | 15.0 | 35.0 |
| | 8 | 0 | traces | traces | 7.5 |
| | 12 | 0 | 0 | 0 | 0 |
| Y671/6 | 0 | 9.8 | 29.5 | 43.0 | 51.2 |
| | 4 | 0.7 | 6.2 | 9.8 | 27.2 |
| | 8 | 0.03 | 0 | traces | 2.5 |
| | 12 | 0 | 0 | 0 | 0 |

Table 7. Growth of *S. occidentalis* strains in $M_0$ minimal medium (1) and in natural medium (potato extract) (2) after 17 h of shaker culture

| Strain | Medium | Dry mass (g/dm³) or number of cells (CFU/cm³) | Utilization of reducing sugars (%) | T (h) | u ($h^{-1}$) | Activity of | | $Y_p$ |
|---|---|---|---|---|---|---|---|---|
| | | | | | | α-amylase | glucoamylase (U/cm³) | |
| Y671/6 | 1 | 7.5 | 76.5 | 4.05 | 0.170 | 2.78 | 66.7 | 0.75 |
| | 2 | $2.85 \times 10^8$ | | 1.38 | 0.500 | 10.6 | 62.0 | |
| Y671/DG/8 | 1 | 7.2 | 72.3 | 2.34 | 0.295 | 2.8 | 63.3 | 0.72 |
| | 2 | $2.63 \times 10^8$ | | 1.05 | 0.657 | 12.1 | 64.4 | |
| Y671 (parent strain) | 1 | 6.3 | 72.3 | 2.77 | 0.249 | 3.05 | 61.7 | 0.63 |
| | 2 | $2.42 \times 10^8$ | | 1.05 | 0.394 | 13.3 | 58.8 | |

$Y_p$ = dry mass/initial starch concentration

Table 8. Growth and activity of *S. occidentalis* strains after 6 months of storage in laboratory conditions in YPS medium at 4°C

| Strain | Dry mass after 17h of culture (g/dm³) | Dry mass increase after storage (%) | u ($h^{-1}$) | T (h) | Activity of | | Increase of activity after storage of | |
|---|---|---|---|---|---|---|---|---|
| | | | | | α-amylase (U/cm³) | glucoamylase (U/cm³) | glucoamylase (%) | α-amylase (%) |
| Y671/6 | 7.5 | 25.0 | 0.170 | 4.05 | 2.78 | 66.7 | 29.2 | -75.6 |
| Y671/DG/8 | 7.2 | 26.3 | 0.295 | 2.34 | 2.80 | 63.3 | 22.6 | -76.2 |
| Y671 (parent strain) | 6.3 | 12.5 | 0.249 | 2.77 | 3.05 | 61.7 | 59.8 | -55.9 |

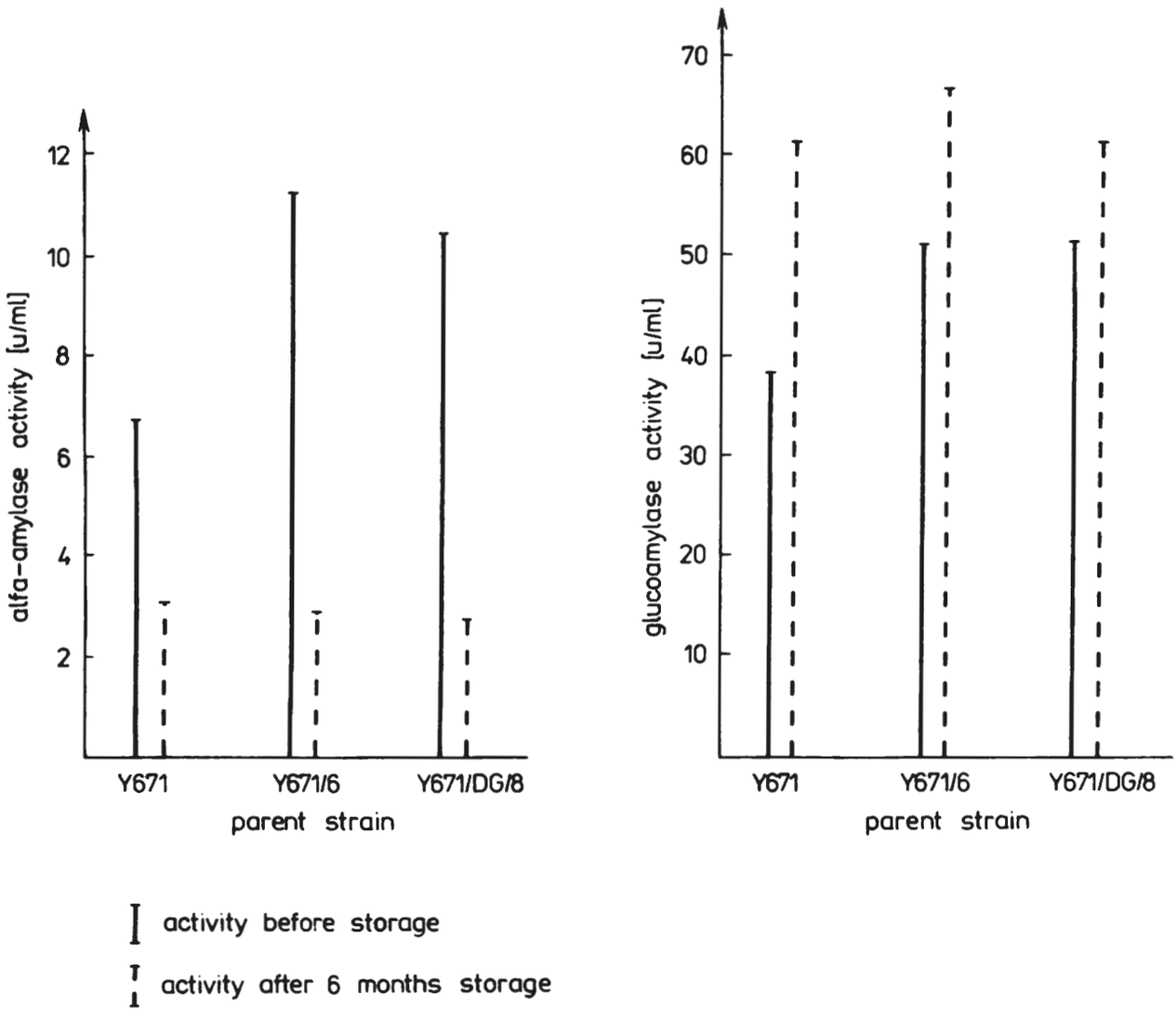


Fig. 3. Amylolytic activity of *S. occidentalis* after 6 months of storage in laboratory conditions

revealed that during storage there occurs a selection of individuals characterized by c. 25% greater biomass production, a 23-29% increase of glucoamylase activity, and a clearly lower α-amylase activity, as compared to the period directly after mutation (Tables 5 and 8). These results indicate the need for research into methods of storing the mutants that would ensure complete stability of the amylolytic complex.

## CONCLUSIONS

1. UV irradiation and 2-deoxyglucose (2-DG) may be used to select *S. occidentalis* clones with increased amololytic activity (from 17 to 65% in the case of α-amylase, and from 5 to 34% in the case of glucoamylase).

2. The high productivity of the mutants, expressed by the biomass yield $Y_p$ of 0.72-0.75, and the up to 76% utilization of starch in synthetic and natural media, show the selected strains to be useful in enriching natural culture media in protein, especially potato media (Table 7, Fig. 2).

3. Mutants stored for six months in standard laboratory conditions displayed 23-29% increases of glucoamylase activity, and high biomass yield

($Y_p$ = 0.72-0.75). The α-amylase activity after storage was clearly lower in comparison to the activity immediately after mutation. This indicates the need for a storage method that would keep this activity stable as well (Table 8).

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## AKTYWNOŚĆ AMYLOLITYCZNA MUTANTÓW *SCHWANNIOMYCES OCCIDENTALIS*

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### S t r e s z c z e n i e

Pod działaniem promieni UV (rys. 1) otrzymano mutanty amylolitycznych drożdży *S. occidentalis* Y671 wyizolowane po odpowiednich wysiewach selekcyjnych (tab. 3). Wytypowano 8 odmian cechujących się zróżnicowanym stosunkiem średnicy strefy hydrolizy skrobi w podłożu agarowym do średnicy kolonii q = 3,2-4,4 (tab. 4). W warunkach hodowli wstrząsanej tworzyły one od 0,1 do 0,4 g/dcm$^3$ więcej biomasy niż szczep macierzysty. Aktywność α-amylazy w tych klonach wahała się od 6,55 do 11,4 j./cm$^3$, a glukoamylazy od 34 do 51,6 j./cm$^3$. Podobną aktywność amylolityczną wykazywały mutanty oporne na 2DG (tab. 5). Wśród ocenianych mutantów aktywność α-amylazy wzrosła od 17 do 65%, a glukoamylazy od 5 do 34% w stosunku do aktywności charakteryzujących szczep macierzysty. Porównawcza ocena wzrostu najaktywniejszego mutanta Y671/6 i szczepu rodzicielskiego Y671 w podłożu $M_0$ z dodatkami od 1 do 5% skrobi wykazała większą dynamikę wzrostu mutanta nawet przy 5% stężeniu skrobi w podłożu (tab. 6, rys. 2) oraz jego wyższą produkcyjność (tab. 7). Podobne tendencje potwierdzono również w podłożu naturalnym, ziemniaczanym (tab. 7). Po 6 miesiącach przechowania mutantów w standardowych warunkach, w populacjach reaktywowanych ze stanu anabiozy i hodowanych w podłożu minimal-

nym $M_0$ + 1% skrobi, aktywność glukoamylazy wzrosła od 23 do 29%, wydajność biomasy o ok. 25% w stosunku do wartości uzyskiwanych bezpośrednio po mutacji, obniżyła się natomiast aktywność α-amylazy (tab. 8,).

Otrzymane wyniki wykazują możliwość stosowania promieni UV do podwyższania aktywności amylolitycznych *S. occidentalis*. Wskazują również na potrzebę dopracowania odpowiednich metod przechowywania mutantów w celu stabilizacji w nich aktywności α-amylazy.