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ACID-FORMING ACTIVITY OF *ASPERGILLUS NIGER* AND AMYLOLYTIC ACTIVITY OF *ASPERGILLUS ORYZAE* AFTER STORAGE IN VARIOUS MEDIA

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Key words: method of storing conidia, acid forming activity, amylolytic activity, *A. niger*, *A. oryzae*.

Industrial strains of *Aspergillus niger* and *Aspergillus oryzae* were comparatively stored under laboratory conditions in distilled water, physiological NaCl solutions, calcium citrate, sand, paraffine oil, and under standard conditions. It was shown that the applied method of storing conidia in distilled water could be utilized in laboratories for preservation of *A. niger* strains, active in production of citric acid and of *A. oryzae*, revealing amylolytic properties.

During the storage in laboratory conditions, industrial strains generally undergo quick degeneration. The preservation of their full production capacities requires continuous application of pure cultures deriving from the suitable storage conditions.

For storage of fungal cultures, the cultivations on solid media, under paraffine oil are recommended, as well as storing the conidia in soil, active carbon [9, 12, 14]; lyophilisation of conidia or mycelium [10] and storage in a liquid nitrogen [4, 16].

Ginnis et al [5] described simple and cheap method for preservation of fungi, yeasts and actinomyces in distilled water. They utilized findings of Castelani [2] that certain pathogenic fungi and yeasts stored for 12 months in distilled water did not reveal in morphological and physiological properties. Benedek [5] modified Castelani's method, applying physiological NaCl solution instead of water.

The recommended methods for preservation of strains are, as a rule, elaborated for specific microorganisms and they may not reveal full

suitability for other physiological groups. Hence, the purpose of the present studies was to examine the influence of storage of conidia of *A. niger* and *A. oryzae* in various media on the acidifying and amylolytic properties of these strains.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

For the experiments, the following material was used: strains of *A. niger* R 8 and R65/4 — morphological mutants, obtained as a result of selection on the molasses media, cultivated in the Institute of Fermentation Technology and Microbiology of Technical University in Łódź and *A. oryzae* E 5, E14, and IAM 3645, deriving from the collection of Pure Cultures of the above Institute.

Sporulating media

The culture of strains *A. niger* was carried out at 30°C, for 14 days on malt wort slants, Czapek-Dox medium [1] and molasses wort [6, 8] while *A. oryzae* cultures were grown on potatoe agar [1].

FERMENTATION MEDIA

The evaluation of acid-forming properties of *A. niger* R8 and R65/4 was conducted in 100cc of molasses medium [8] by a surfacial method, in Erlenmeyer flasks with capacity 300 cm^3, for 12 days. On the 8th, 10th and 12th day of fermentation, changes in the total acidity and the level of dry matter of mycelium, were determined. The batch culture of strains *A. oryzae* was carried out for 8 days at 30°C in 20 cm^3 of synthetic medium with the composition given by Skinka and Chakrabarty [13].

STORAGE OF *A. NIGER* AND *A. ORYZAE*

The examined strains were stored at room temperature for 1 year on agar slants, in paraffine oil and in a form of conidial suspension in distilled water, in physiological NaCl solution, in calcium citrate and in sand. The density of the suspension in the examined media was established on the level 10^5-10^6 conidia/cm^3. After the protection from drying or moisturizing, the samples constituted the material for studies on the surival of conidia and acid-forming and amylolytic properties [5, 12].

EVALUATION OF SURVIVAL OF CONIDIA

The survival of conidia suspended in a distilled water and in physiological NaCl solution were examined by the plate method on malt wort 7°Blg. The seeds were made from several dilutions in 5 parallel trials. Number of surviving cells was given as amount of cell units capable of growth in a form of colonies (CFU/cm^3 — colonies-forming units).

EVALUATION OF AMYLOLYTIC ACTIVITY

The amylolytic activity was determined in the post-fermentation liquid by the method described by Ruchladeva and Goriecheva [11]. The amount of starch decomposed in the process of enzymatic reaction was determined.

OTHER DETERMINATIONS

Total acidity was determined by titration of 2 cm^3 of post-fermentation liquid with 0.1 n NaOH agains pnenolophtalein. Dry matter of mycelium was determined by weight, after drying the sample at 103-105°C to the constant weight. The results were given in g/100 cm^3 of medium [15]. Reducing sugars were determined by the method of Luff-Schoorl, expressed in glucose [3].

RESULTS

ACID-FORMING ACTIVITY OF *A. NIGER* R8 AND R65/4 DURING THE STORAGE IN A DISTILLED WATER AND IN PHYSIOLOGICAL NaCl SOLUTION

Conidia of *A. niger* R8 and R65/4 were stored for 1 year in a distilled water and in physiological solution of NaCl. During the storage period, their acidifying properties and survival rate after 2, 5, 10 and 12 months were determined. The results of the studies are presented in Table 1 and 2 and in Fig. 1.

Table 1. Fermentation abilities of strains: *A. niger* R8 and R65/4 stored in distilled water for 1 years

Strain	Determinations	Days of fermentation	Period of storing conidia in a distilled water (months)				
			0	2	5	10	12
R8	total acidity (cm^3 0.1 n NaOH/2 cm^3 of medium)	8	36.6	31.3	28.9	22.5	16.2
		10	36.8	43.2	35.1	32.6	19.8
		12	35.9	32.2	36.2	35.0	21.4
	dry matter of mycelium (g/100 cm^3)	8	1.1386	0.8704	1.1346	0.7115	0.5504
		10	1.2041	1.2370	1.3645	0.8570	0.6480
		12	1.1957	1.0317	1.4228	1.1270	0.6989
R65/4	total acidity (cm^3 0.1 n NaOH/2 cm^3 of medium)	8	29.0	31.8	30.6	16.0	16.5
		10	33.4	34.2	35.2	30.3	20.2
		12	33.2	37.6	36.9	34.6	25.2
	dry matter of mycelium (g/100 cm^3)	8	1.0513	1.1772	1.1930	0.5624	0.5224
		10	1.0905	1.3190	1.3233	0.8060	0.6297
		12	1.1002	1.1626	1.4131	0.9890	0.6367

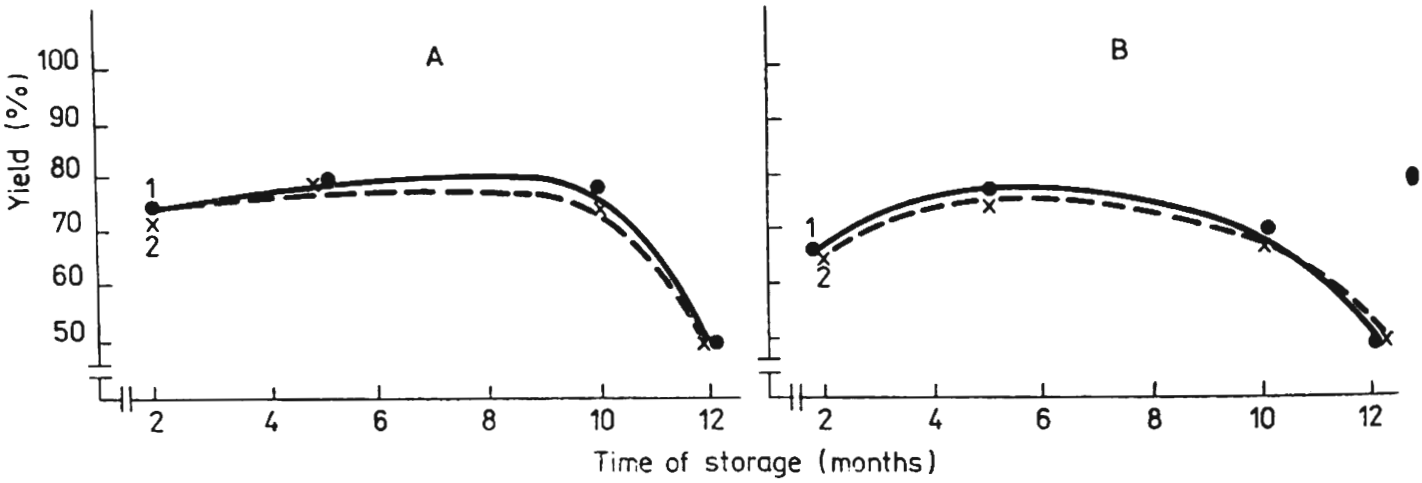

Fig. 1. Yield of citric acid fermentation of strains: *A. niger* R8 and R65/4 during their storage in a distilled water (A) and in physiological solution of NaCl (B) (10th day of fermentation); *1* — strain R8, *2* — strain R65/4

The analysis of acidifying properties of these strains revealed that during the period of up to 10 months, they retained high activity and on the 10th day of fermentation, acidity of media reached 30-32.6 cm^3 0.1 n NaOH/2 cm^3 of medium (Table 1). After 12 months of storing conidia in a distilled water, an evident lowering in acidifying ability was observed (by about 61%). It was simultaneously connected with almost double decrease in biomass yield. The strains grew on a whole surface of fermentation liquid in a form of thin, slightly corrugated mycelium. It was also observed that the stored strains showed delayed accumulation of acids in

Table 2. Fermentation abilities of *A. niger* R8 and R65/4 stored in physiological NaCl solution for 1 year

Strain	Determinations	Days of fermentation	Period of storing conidia in physiological NaCl solution (months)				
			0	2	5	10	12
R8	total acidity (cm^3 0.1 n NaOH/2 cm^3 of medium)	8	36.6	29.8	29.8	22.0	16.8
		10	36.8	30.9	34.7	30.3	20.2
		12	35.9	37.2	36.4	33.8	24.6
	dry matter of mycelium (g/100 cm^3)	8	1.1386	1.1275	1.1486	0.7215	0.6207
		10	1.2041	1.2762	1.3961	0.8280	0.6925
		12	1.1957	1.1564	1.4660	0.9685	0.8437
R65/4	total acidity (cm^3 0.1 n NaOH/2 cm^3 of medium)	8	29.0	29.8	30.0	20.3	19.6
		10	33.4	30.1	35.0	28.4	21.6
		12	33.2	39.6	36.0	33.5	25.8
	dry matter of mycelium (g/100 cm^3)	8	1.0513	1.2148	1.2100	0.7080	0.6194
		10	1.0905	1.2526	1.2900	0.7600	0.6847
		12	1.1002	1.1462	1.4020	1.1140	0.7987

the substrate. During the first months of storage, on the 8th day of fermentation, high value of total acidity equal to 31.8 cm^3 0.1 NaOH was obtained and after 12 months it amounted to about 16 cm^3 0.1 n NaOH/ /2 cm^3 of the medium (Table 1). In the fermentation cycle prolongated to 12 days, the total acidity was increased to 25.2 cm^3 0.1 n NaOH.

A. niger R8 and R65/4 fermented with the similar yield (Fig. 1). The yield, calculated in relation to sugar present in the medium, during 10-month — storage was found within the limits 73-78%, being lowered after 12 months to about 50%. As it can be seen from Table 2 and Fig. 1, during the storage in the physiological NaCl solution, mutants of *A. niger* behaved in the analogical way as during their storage in a distilled water (Table 1). Up to 10 months of storage, they did not loose their activity. The prolongation of the storage to 12 months caused impairement of productive abilities of these strains. The yield of fermentation process (Fig. 1) during 10 months was found in the limits 66-78% and after 12 months it was distinctly lowered, reaching to 52%.

ACID-FORMING ACTIVITY OF *A. NIGER* R8 AND R65/4 DURING THE STORAGE IN CALCIUM CITRATE AND IN STERILE SAND

The acidifying activity of *A. niger* R8 and R65/4 stored in calcium citrate and in sterile sand, was determined after 2, 5, 8, 10 and 12 months (Table 3 and 4). To the 8th month, the strains maintained their initial activity, carrying out the critic acid fermentation with the yield = 76% (Fig. 2). The impairement of acidifying properties had place no earlier than after 10 months of storage. The yield of fermentation process was lowered to about 60%. The same run was observed after 12 months of storage (Fig. 2).

The populations, originating from inoculum stored in calcium citrate

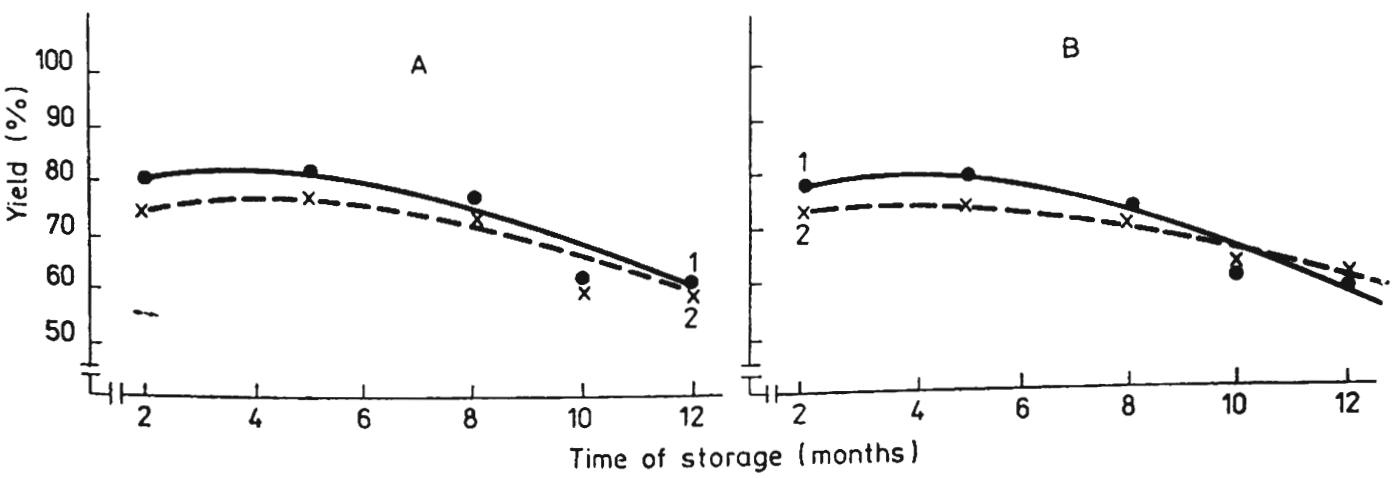

Fig. 2. Yield of citric acid fermentation of strains: *A. niger* R8 and R65/4 during their storage in calcium citrate (A) and in a sterile sand (B) (10th day of fermentation); *1* — strain R8, *2* — strain R65/4

and sand, were characterized by the similar acidifying activity (Table 3 and 4). It is concluded that calcium citrate and sand are equally suitable valuable media for storage of *A. niger* conidia.

Table 3. Fermentation abilities of strains: *A. niger* R8 and R65/4 stored in calcium citrate

Strain	Determinations	Days of fermentation	Time of storing the cinidia in calcium citrate (months)				
			2	5	8	10	12
R8	total acidity (cm^3 0.1 n NaOH/2 cm^3 of medium)	8	26.1	24.8	23.6	19.9	20.5
		10	38.3	34.0	30.8	24.9	25.5
		12	37.5	34.6	34.0	25.1	26.2
	dry matter of mycelium (g/100 cm^3)	8	0.9710	1.0376	0.5034	0.8043	0.8273
		10	1.0925	1.2205	0.8574	1.9894	1.0262
		12	1.3020	1.4603	1.0170	1.0371	1.1840
R65/4	total acidity (cm^3 0.1 n NaOH/2 cm^3 of medium)	8	27.4	26.0	17.3	19.4	21.8
		10	34.3	32.3	29.6	23.4	25.7
		12	36.0	35.7	33.4	24.4	26.3
	dry matter of mycelium (g/100 cm^3)	8	1.0032	1.1274	0.6760	0.7509	0.7752
		10	1.0043	1.1724	0.7620	0.9874	1.0567
		12	1.2988	1.4251	0.9910	1.0426	1.2460

Table 4. Fermentation abilities of strains: *A. niger* R8 and R65/4 stored in a sterile sand

Strain	Determinations	Days of fermentation	Time of storing the conidia in a sterile sand (months)				
			2	5	8	10	12
R8	total acidity (cm^3 0.1 n NaOH/2 cm^3 of medium	8	27.5	25.4	18.6	18.4	22.4
		10	36.1	33.2	28.6	23.6	24.4
		12	34.9	35.0	32.0	24.3	25.8
	dry matter of mycelium (g/100 cm^3)	8	0.9869	1.1915	0.6900	0.7476	0.8152
		10	1.0238	1.2366	0.8143	0.9327	1.0112
		12	1.3193	1.4853	1.2245	0.9556	1.1437
R65/4	total acidity (cm^3 0.1 n NaOH/2 cm^3 of medium	8	25.2	24.0	18.0	20.7	20.4
		10	33.3	30.5	28.6	23.5	25.8
		12	36.7	34.5	32.0	24.2	26.4
	dry matter of mycelium (g/100 cm^3)	8	0.9520	1.1387	0.7475	0.8277	0.7694
		10	1.0112	1.2020	1.0089	0.8903	1.1679
		12	1.3133	1.5052	1.2840	0.9957	1.1884

ACID-FORMING ACTIVITY OF *A. NIGER* R8 AND R65/4 DURING THE STORAGE IN PARAFFINE OIL AND IN STANDARD CONDITIONS

Mutants of *A. niger* R8 and R65/4, stored for 12 months at room temperature on agar skants of malt wort, of molasses wort, on Czapek-Dox medium and on the above mentioned substrates in paraffine oil, were evaluated after 2, 5, 8 and 12 months. The results of the studies are presented in Fig. 3 and Table 5. The acid-forming properties of *A. niger* mutants were not dependent on the type of culture medium. The populations deriving from conidia collected from various growth media produced citric acid with the similar yield (Fig. 3).

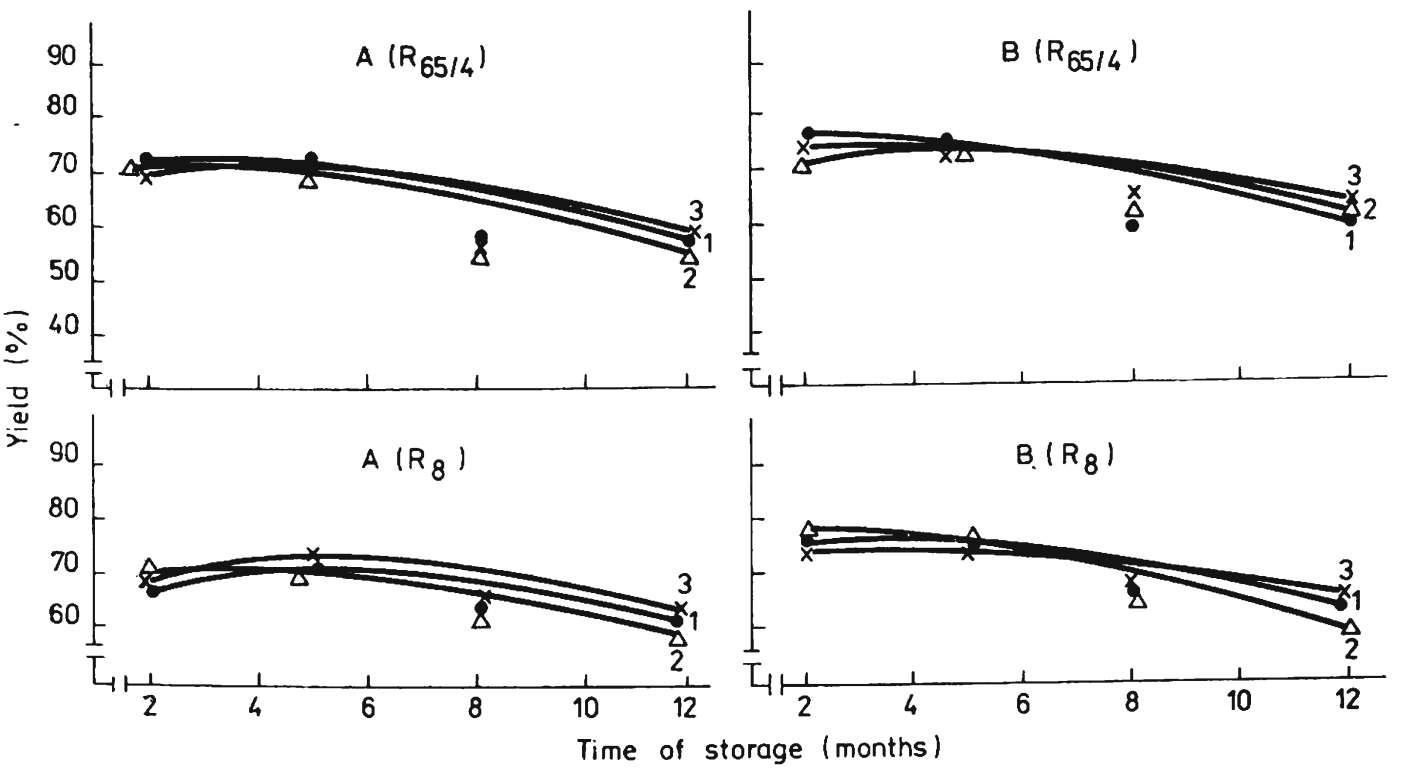

Fig. 3. Yield of citric acid fermentation of strains: *A. niger* R8 and R65/4 during their storage on agar skews (A) and under paraffine oil (B); *1* — slants of malt wort, *2* — slants of Czapek-Dox medium, *3* — slants of molasses wort

After 2-month-storage of agar slants at room temperature, the fermentation yield of *A. niger* R8 and R65/4 was found in the range 67.5-72% and after 8 months, the decrease to value 55.2-63.0% was stated. After 12 months of storage of the strains, no further impairement of their production abilities was observed (Fig. 3). The corresponding populatons, deriving from the conidia stored in paraffine oil were characterized by a slightly higher acid-forming activity (Fig. 3, Table 5).

From the comparison of the storage methods applied (Fig. 4 and 5) it results that *A. niger* strains most quickly undergo degeneration during the storage on agar slants. Cultures stored for the same time (8 months) in calcium citrate conducted fermentation process with the yield about 76%.

Table 5. Acid-forming properties of strains: *A. niger* R8 and R65/4 stored in paraffine oil and on agar slants (10 days of fermentation

Strain	Sporulating medium	Time of storing the conidia (months)							
		2		5		8		12	
		agar slants	agar slants under paraffin oil	agar slants	agar slants under paraffin oil	agar slants	agar slants under paraffin oil	agar slants	agar slants under paraffin oil
R8	wort	29.2	39.8	36.9	37.4	25.4	27.1	29.8	30.3
	molasses wort	29.4	33.6	35.4	35.4	23.5	26.2	31.0	31.0
	Czapek-Dox	29.8	40.1	36.5	38.3	21.4	24.9	28.0	28.4
R65/4	wort	22.1	40.7	37.5	37.6	23.6	20.5	27.6	28.2
	molasses wort	26.3	36.6	37.5	35.8	20.9	26.1	27.9	29.0
	Czapek-Dox	29.9	37.1	36.8	37.6	22.1	25.6	26.4	28.4

Results of total acidity is given in cm^3 0.1 n NaOH/2 cm^3 of medium.

After 12-months storage in the discussed media, mutants of *A. niger* R8 and R65/4 maintained the highest production activity during the storage in calcium citrate and in sand (Fig. 4 and 5).

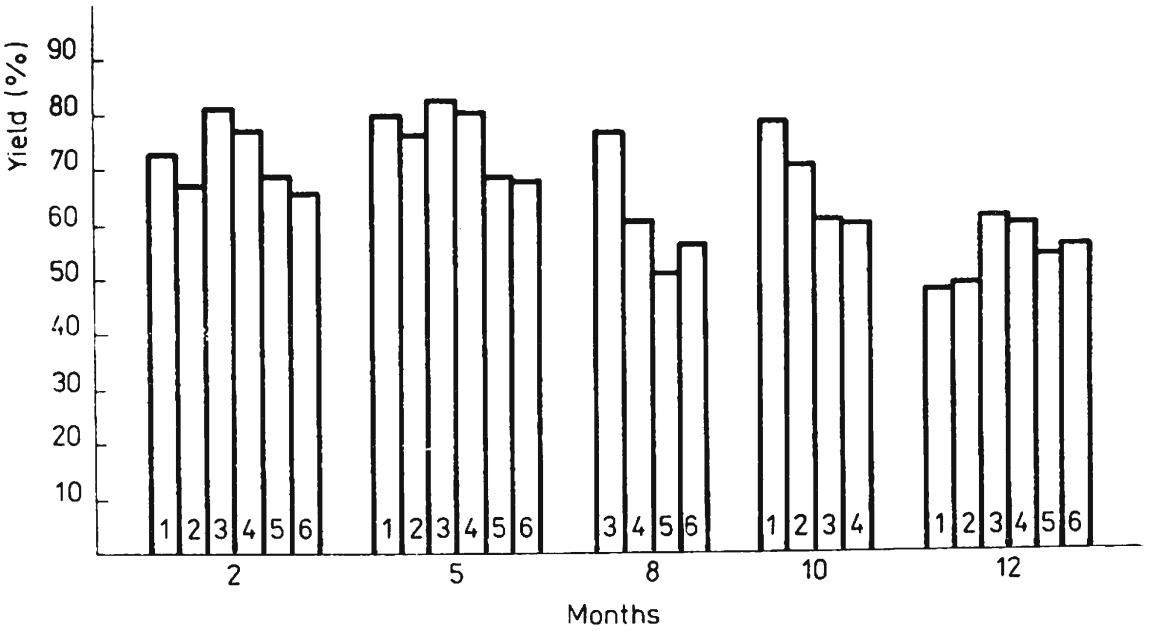

Fig. 4. Influence of storage conditions on the run of citric fermentation of strain R8 (10th day of fermentation); *1* — distilled water, *2* — physiological NaCl solution, *3* — calcium citrate, *4* — sterile sand, *5* — agar slant of molasses wort, *6* — agar slant of molasses wort under paraffine oil

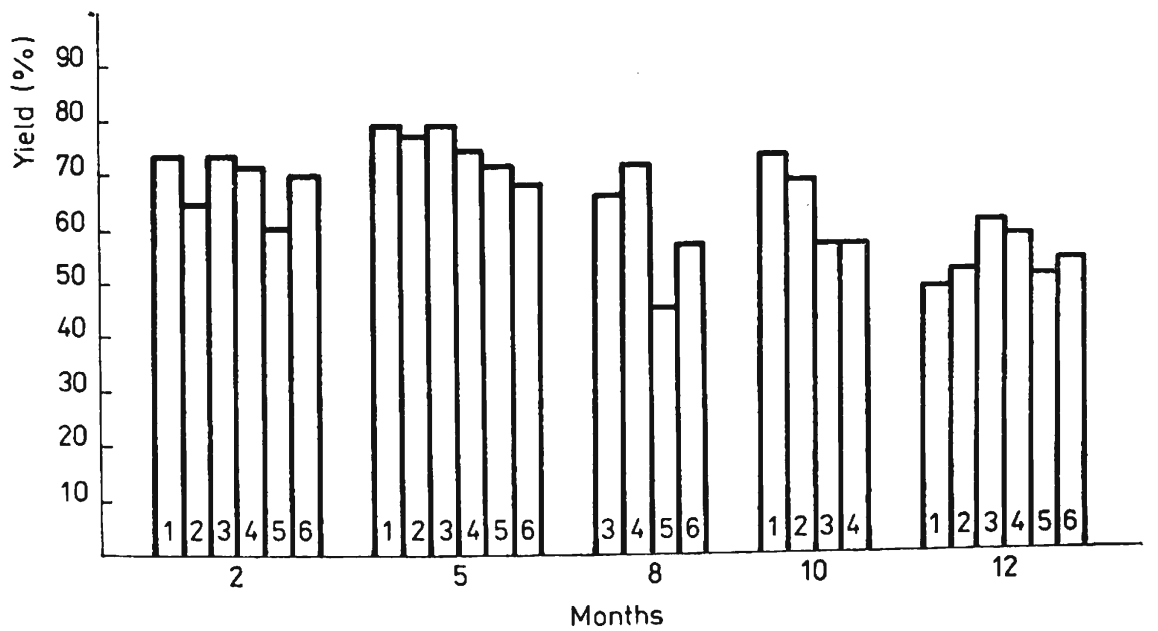

Fig. 5. Influence of storage conditions on the run of citric fermentation of strain R65/4 (10th day of fermentation); *1* — distilled water, *2* — physiological NaCl solution, *3* — calcium citrate, *4* — sterile sand, *5* — agar slant of molasses wort, *6* — agar slant of molasses wort under paraffine oil

THE AMYLOLYTIC ACTIVITY OF *A. ORYZAE* DURING THE STORAGE IN VARIOUS ENVIRONMENTAL CONDITIONS

From Fig. 6, it results that strains of *A. niger oryzae* E5, E14 and IAM 3645 were characterized by the similar amylolytic properties. The maximal quantities of hydrolyzed starch were found in the range of 71.2-77.7 mg/

/cm³. Cultures stored in a distilled water for 7 months retained the amylolytic activity similar to that one of the initial samples. The prolongation of storage time to 12 months caused the weakening of amylolytic activity which amounted then to 14-18% for the examined strains (Fig. 6).

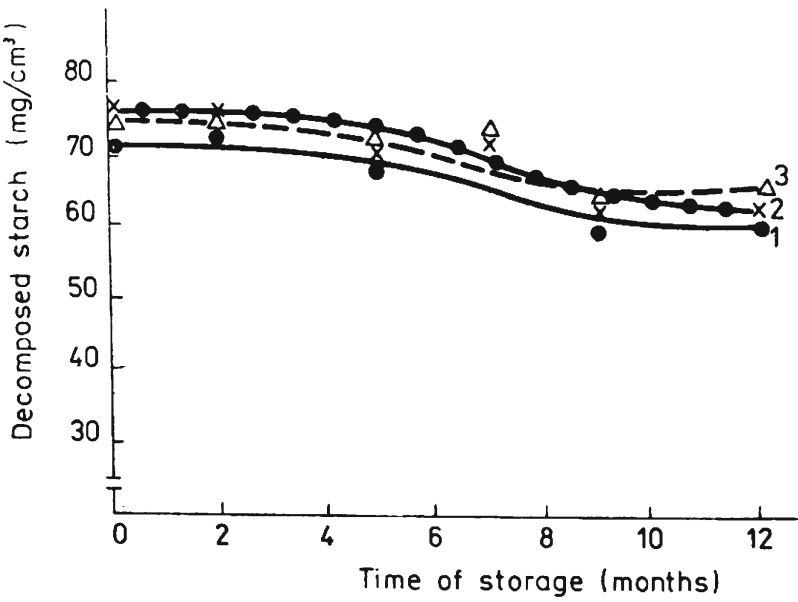

Fig. 6. Amylolytic activity of strains: *A. oryzae* during the storage in distilled water (4th day of cultivation); *1* — *A. oryzae* E5, *2* — *A. oryzae* E14, *3* — *A. oryzae* IAM 3645

Conidia of *A. oryzae* E5 stored for 10 months in calcium citrate revealed lowering of amylolytic activity to 65 mg of hydrolyzed starch (cm³ of fluid (Fig. 7). The populations stored in paraffin oil (Fig. 7) preserved high amylolytic activity of conidia to the 8th month of storage. During the prolongated period of storage (up to 12 months), the 26% — decrease in amylolytic activity of the discussed strain was stated. It may be concluded that for *A. oryzae* E5, calcium citrate and distilled water are more suitable storage media than paraffin oil.

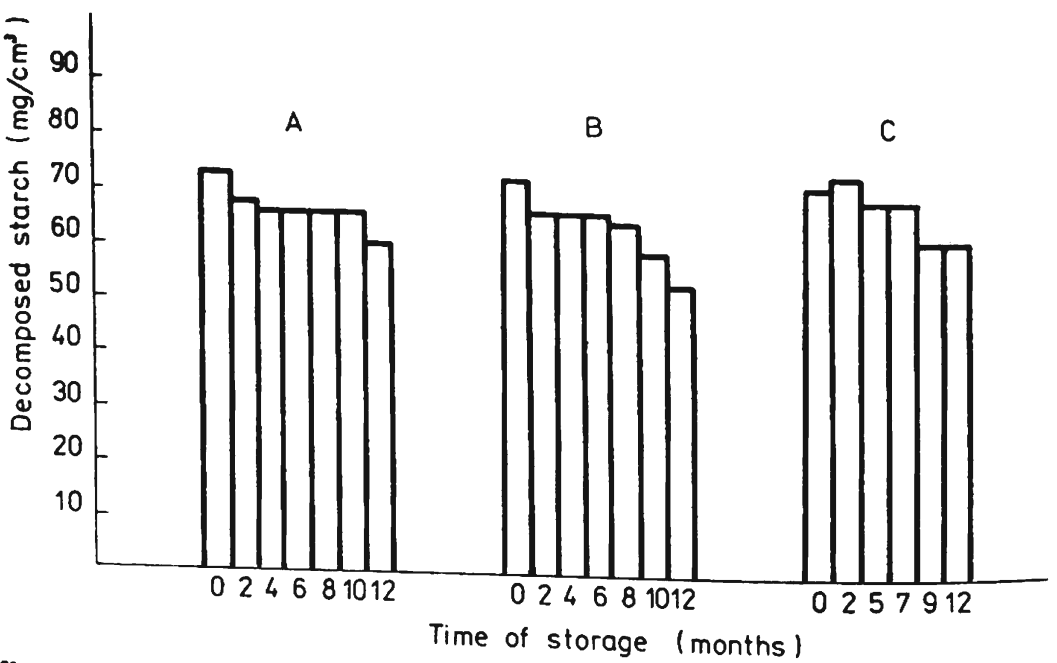

Fig. 7. Influence of storage conditions on the amylolytic activity of *A. oryzae* E5; *A* — calcium citrate; *B* — paraffin oil, *C* — distilled water

SURVIVAL OF CONIDIA

The comparison of survival of conidia of *A. niger* R8 and R65/4 and of *A. oryzae* stored in a distilled water and in physiological NaCl solution, is presented in Fig. 8. From Fig. 8 it may be concluded that water is more suitable environment for storage of *A. niger* R8 and R65/4 conidia than physiological solution of NaCl. The survival of the examined strains in a distilled water after 2-month storage remains almost unchanged (98.6-100%) while in the physiological NaCl solution, the survival of conidia was decreased by 6-20%.

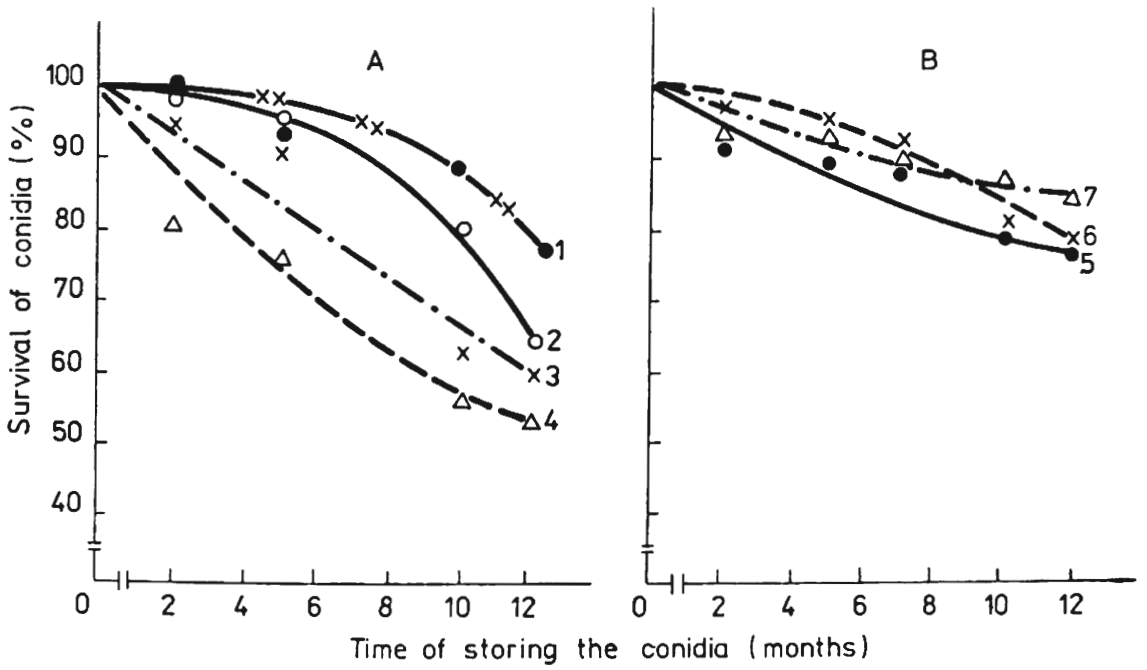

Fig. 8. Comparison of survival rate of conidia of *A. niger* (A) and *A. oryzae* (B) during their storage in a distilled water and in physiological NaCl solution; *1* — R8 stored in water, *2* — R65/4 stored in water, *3* — R8 stored in physiological solution of NaCl, *4* — R65/4 stored in physiological solution of NaCl, *5* — *A. oryzae* E5 stored in water, *6* — *A. oryzae* E14 stored in water, *7* — *A. oryzae* IAM 3645 stored in water

After 1-year storage of strains R8 and R65/4 in a distilled water, conidia survived in 64-78% and in the physiological NaCl solution — in 53.3-59.2% (Fig. 8). The lower survival of conidia stored in physiological solution of NaCl did not exert, however, the influence upon the acidifying properties of populations deriving from this storage medium (Table 2, Fig. 1).

As it can be seen from Fig. 8, strains *A. oryzae* E5, E14, IAM 3645 were characterized by a high survival of conidia in a distilled water. After 7-month-storage, value for the survival of conidia (88-94.6%) was similar to that one for the initial period of storage. During the examined period, the highest survival = 94.6% was shown by *A. oryzae* E14. After 12 month storage the survival of conidia was decreased only by 11%.

The obtained results allow to consider the distilled water as a suitable environment for storage of *A. niger* and *A. oryzae* strains; the more so,

as according to the literature data [7, 17] the stimulation of the inactive conidium for germination requires activation of enzymes what, in case of *A. niger*, is connected with the presence of sugar (glucose), nitrogen source, phosphorus and CO_2.

CONCLUSIONS

1. Industrial strains of *A. niger* for production pruposes may be stored in laboratory conditions in distilled water, physiological NaCl solution, in calcium citrate and in sand.

2. The maximal time for storage of conidia ensuring the production stability of citric acid is differentiated;

a) in distilled water and physiological NaCl solution, conidia of *A. niger* R8 and R65/4 did not loose the acidforming activity to 10 months,

b) in calcium citrate and in sand, mutants of *A. niger* R8 and R65/4 maintained the initial activity during 8-month storage,

c) the time ensuring high acid-forming activity of *A. niger* conidia stored on agar slants and in paraffine oil was shorter than 8 months.

3. Distilled water is a suitable environment for storage of conidia of *A. niger* R8 and R65/4 and *A. oryzae* E5, E14 and IAM3645. After 1-year storage of R8 and R65/4 in distilled water the conidia survived in 64-78%. *A. oryzae* strains revealed high survival rate of conidia in 77-84% and slight decrease in activity.

4. Introduction of conidia to physiological NaCl solution after 1-year storage showed the lowering in their survival to value 53-59%. Lower survival rate of conidia in the discussed medium did not influence the acidifying properties of the resultant populations.

5. The amylolytic activity of *A. oryzae* E5 was unchanged, irrespectively of the storage environment during 7 months. The prolongation of storage time to 12 months caused decrease in amylolytic activity of this strain (14-26%). The lowest amylolytic activity was stated in case of *A. oryzae* E5 stored in paraffin oil.

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AKTYWNOŚĆ KWASOTWÓRCZA *A. NIGER* ORAZ AKTYWNOŚĆ AMYLOLITYCZNA *A. ORYZAE* PO PRZECHOWANIU W RÓŻNYCH ŚRODOWISKACH

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Streszczenie

Konidia szczepów produkcyjnych *A. niger* oraz *A. oryzae* przechowywano w warunkach laboratoryjnych w różnych środowiskach: w wodzie destylowanej, w roztworze fizjologicznym NaCl, w cytranianie wapnia, w piasku, pod olejem parafinowym oraz w warunkach standardowych na skosach agarowych.

Badano przeżywalność (rys. 8) oraz oceniano zdolności fermentacyjne i aktywności amylolityczne szczepów (tab. 1-5; rys. 1-7). Szczepy *A. niger* przechowywane w wodzie destylowanej i w roztworze fizjologicznym NaCl zachowały wysoką stabilność produkcyjną do 10 miesięcy (tab. 1, 2; rys. 1). W cytrynianie wapnia i w piasku wysokie zdolności kwaszące (ok. 30 cm^3 0,1 n NaOH/2 cm^3 podłoża) uzyskano do 8 miesiąca przechowywania (tab. 3, 4; rys. 2). W pozostałych środowiskach czas zabezpieczający niezmienioną aktywność konidiów był krótszy niż 8 miesięcy (rys. 3; tab. 5). Szczepy *A. oryzae* przechowywane w wodzie destylowanej w czasie 1 roku wykazały spadek aktywności amylolitycznej od 14 do 18% (rys. 6).

Zastosowana metoda przechowywania konidiów w wodzie destylowanej może być wykorzystana w laboratoryjnym przechowalnictwie szczepów *A. niger* czynnych w produkcji kwasu cytrynowego oraz *A. oryzae* wykazujących uzdolnienia amylolityczne.