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V.S. Pidgorskyi, O.D. Ianieva, M.O. Fomina, K.S. Tkachenko, V.S. Stoyan

Zabolotny Institute of Microbiology and Virology,
National Academy of Sciences of Ukraine,
154 Acad. Zabolotny Str., Kyiv, 03143, Ukraine

ETHANOL PRODUCTION FROM STARCH BY YEASTS ISOLATED FROM CROPS AND DAIRY PRODUCTS

The aim of this work was to study the ability of yeasts isolated from crops and dairy products to convert starch to ethanol.

The isolated yeasts were screened for their ability to hydrolyze starch. Six most active strains were identified as Lipomyces mesembrius spp. 5.4, 5.5 and 6.4, Shwanniomyces vanrijiae var. yarowii F33, Torulaspora sp. F7 and Candida sp. S26. The selected yeasts produced low levels of ethanol from starch under aerobic conditions – 0.006–0.129 g/l (0.3–0.87 % of theoretical yield) and microaerobic conditions – 0.089–0.35 g/l (1.61–6.07 % of theoretical yield). These amylolytic yeast strains will be studied as the potential candidates for the cocultivation with efficient ethanol producers which do not possess the ability to directly hydrolyze starch.

K e y w o r d s: amylolytic yeasts, ethanol, starch, biofuel.

As the world oil and gas reserves are being depleted the demand for renewable fuels and reduction in green-gas emissions has been increased in the last decades. The development of fuels produced from biomass and waste materials provides the alternative to conventional petroleum fuels. Ethanol is one of the most advantageous and widely used biofuels. [8]. Yeasts are the promising group of unicellular eukaryotes used in many fields of industry and agriculture. Almost 95 % of industrially produced ethanol is obtained by yeast fermentation.

The main feedstocks for bioethanol production are sugary plants (sugar cane, beet, etc.), starchy crops (corn, wheat, potatoes) and lignocellulosic materials (wood, paper, agricultural and industrial waste). The largest ethanol producers USA and Brazil obtain ethanol from starch and sugar–containing crops: sugar cane and corn [3]. Ukraine is among world leading producers of such starchy crops as corn and potatoes. Corn production in 2011 in Ukraine was 19 million ton [1]. As a result of potatoes processing 15–40 % of its weight goes to waste [12]. The co–fermentation of such feedstock with other sugar–containing waste materials, for example whey, could result in the more efficient ethanol yield.

Some yeasts possess the ability to degrade starch and were proposed as the promising candidates for conversion of starch-containing substrates into ethanol and single-cell protein [11]. Few amylolytic yeasts are also capable of direct conversion of starch into ethanol. Yeasts belonging to species *Saccharomyces diastaticus* and *Endomycopsis capsularis* have been shown to produce ethanol from starch without prior substrate treatment with 21–38 % efficiency [14]. Other examples of such amylolytic yeasts are represented *by Endomycopsis fibugilera* [10]. However the use of such amylolytic yeasts for the direct fermentation of starch into ethanol is not economically viable as ethanol yield is too low com-

pared to the processes using *Saccharomyces cerevisiae*. More promising could be the co-fermentation of amylolytic yeasts with saccharomycetous yeasts not able to hydrolyze starch [9, 14].

The purpose of this work was to perform the screening for amylolytic yeasts among strains isolated from crops and dairy products and to determine their ability to produce ethanol from starch.

Materials and methods. The screening for amylolytic yeasts was conducted among yeasts strains isolated from various agricultural crops and dairy products (Table 1). The isolation of yeast strains from crops and dairy products was conducted as follows: 1 g sample was dispersed and dissolved in sterile water. 10-fold dilutions were placed on the medium containing starch as a sole source of carbon. Individual colonies on the plate which represented different morphotypes were examined microscopically to select yeast strains. Each strain was purified by streaking at least 3 times.

Table 1

Sources for isolation of amylolytic yeasts

Fruit	Apple
	Melon
	Pomegranate
Root vegetables and other crops	Beetroot
	Cabbage
	Corn
	Potatoes
Dairy	Cheese
	Sour cream
	Yogurt

Identification of isolated yeast strains was based on their morphological and physiological characteristics. All tests were conducted according to Kurtzman [13].

Preliminary screening of amylolytic yeasts was performed using starch agar containing 6.7 g/l Yeast Nitrogen Base, 20 g/l starch, 20 g/l agar, pH – 5.5. Yeasts were cultivated at 26–28 ^{0}C for 7 days. After that Lugol solution (0.2 % I_2, 2 % KI) was poured on the agar surface detecting the hydrolysis zone or yellow colour of the colonies indicating amylolytic activity. The most active strains were selected for further research.

The ability of the selected strains to assimilate starch and produce ethanol was studied at 28–30 °C under aerobic (207 rpm at the rotary shaker), stationary (incubator without agitation) and microaerobic conditions (in flasks with rubber stoppers to prevent oxygen access) in the medium containing (g/L): starch – 20, peptone – 20, yeast extract – 10, pH 5.0.

Cell growth was determined by measuring sample optical density using photoelectrocolorimeter KFK-2 at 540 nm. The standard curve (cell dry weight vs. optical density) was established.

Ethanol concentration was measured by gas chromatography using "Chrom-5" chromatograph with flame ionization detector with helium as the carrier gas, at 80 ^{0}C, flow rate at 20 ml/min. Ethanol yield was calculated using following equation:

$Y_{P/S}=P / S_0-S_f$, where P – ethanol concentration, g/l, S_0 – intitial starch concentration, g/l, S_f – final starch concentration, g/l.

Starch concentration was estimated using iodine-starch complex test at spectrophotometer CF-26 (590 nm). All tests were performed in triplicate and statistically analyzed.

Results and discussion. Yeasts able to hydrolyze starch are mostly represented by yeasts belonging to species *Schwanniomyces (Debaryomyces) occidentalis, Saccharomyces diastaticus (S. cerevisiae var. diastaticus),* genera *Lipomyces, Candida* etc. [11].

In the present work 25 yeast strains were isolated from fruits, root vegetables and other agricultural crops on the medium containing starch as a sole source of carbon. Most yeasts were isolated from apples (9 strains) and potatoes (5 strains). The preliminary phenotypic identification of the isolated yeasts was conducted (Table 2).

Table 2

Yeast strains isolated from fruits and other crops

Strain	Source	Genus, species	Strain	Source	Genus, species
1.2	beetroot	*Sporobolomyces ogasawarensis*	7.2	melon	*Schwanniomyces vanrijiae var. yarrowii*
1.3	beetroot	NI*	9.2	cabbage	*Rhodotorula* sp.
1.4	beetroot	*Lipomyces mesembrius*	9.3	cabbage	*Debaryomyces hansenii*
5.1	apple	*Hannaella (Cryptococcus) zeae*	9.5	cabbage	*Debaryomyces hansenii*
5.2	apple	*Rhodotorula silvestris*	10.1.1	Potatoes	*Debaryomyces hansenii*
5.3	apple	*Debaryomyces hansenii*	10.1.2	Potatoes	*Debaryomyces hansenii*
5.4	apple	*Lipomyces mesembrius*	10.2	potatoes	*Rhodotorula sp.*
5.5	apple	*Lipomyces mesembrius*	11.1	potatoes	*Debaryomyces hansenii*
6.1	apple	NI*	12.1	potatoes	*Metschnikowia sp.*
6.2	apple	*Rhodotorula* sp.	13.4	corn	*Rhodotorula* sp.
6.3	apple	*Derxomyces* sp.	13.5	corn	*Cryptococcus terrestris*
6.4	apple	*Lipomyces mesembrius*	14.1	corn	*Lipomyces* sp.
7.1	melon	*Debaryomyces hansenii*			

*NI – not identified

Seven isolates were identified as *Debaryomyces hansenii* which is one of the most prevalent yeasts in the environment found in soil, plants, water, foods etc. [4]. Nine yeast strains belonged to basidiomycetes e.g. genera *Cryptococcus, Rhodotorula, Derxomyces, Hanaella, Sporobolomyces*. Basidiomycetous yeasts predominantly inhabit fruits, vegetables, phylloplane, rhizosphere, water sources [5–6]. Two isolates were identified as *Schwanniomyces vanrijiae var. yarrowii* and *Metschnikowia sp.* Five strains belonged to genus *Lipomyces* and four of them were identified as *Lipomyces mesembrius*. Lipomycetes are known for their high amylolytic activity [7]. We were not able to identify the remaining two yeast isolates by conventional methods.

The screening for amylolytic yeasts was also performed among 76 yeast strains isolated from home-made dairy products (sour cream, cheese, yogurt) together with 25 yeast strains isolated from crops mentioned above. Dairy products isolates manifested a much higher number of strains (60 cultures) able to develop the clear halo in starch-containing agar comprising almost 80 % of all tested yeasts isolated from dairy sources (Figs. 1A, B). In contrast,

only 32 % of yeasts isolated from fruits, vegetables and grains (8 out of 25 tested strains) showed the ability to hydrolyze starch (Fig. 1C). In general, 68 strains out of total 101 tested isolates (67 %) were able to form the hydrolysis zone on the starch agar (Fig. 1).

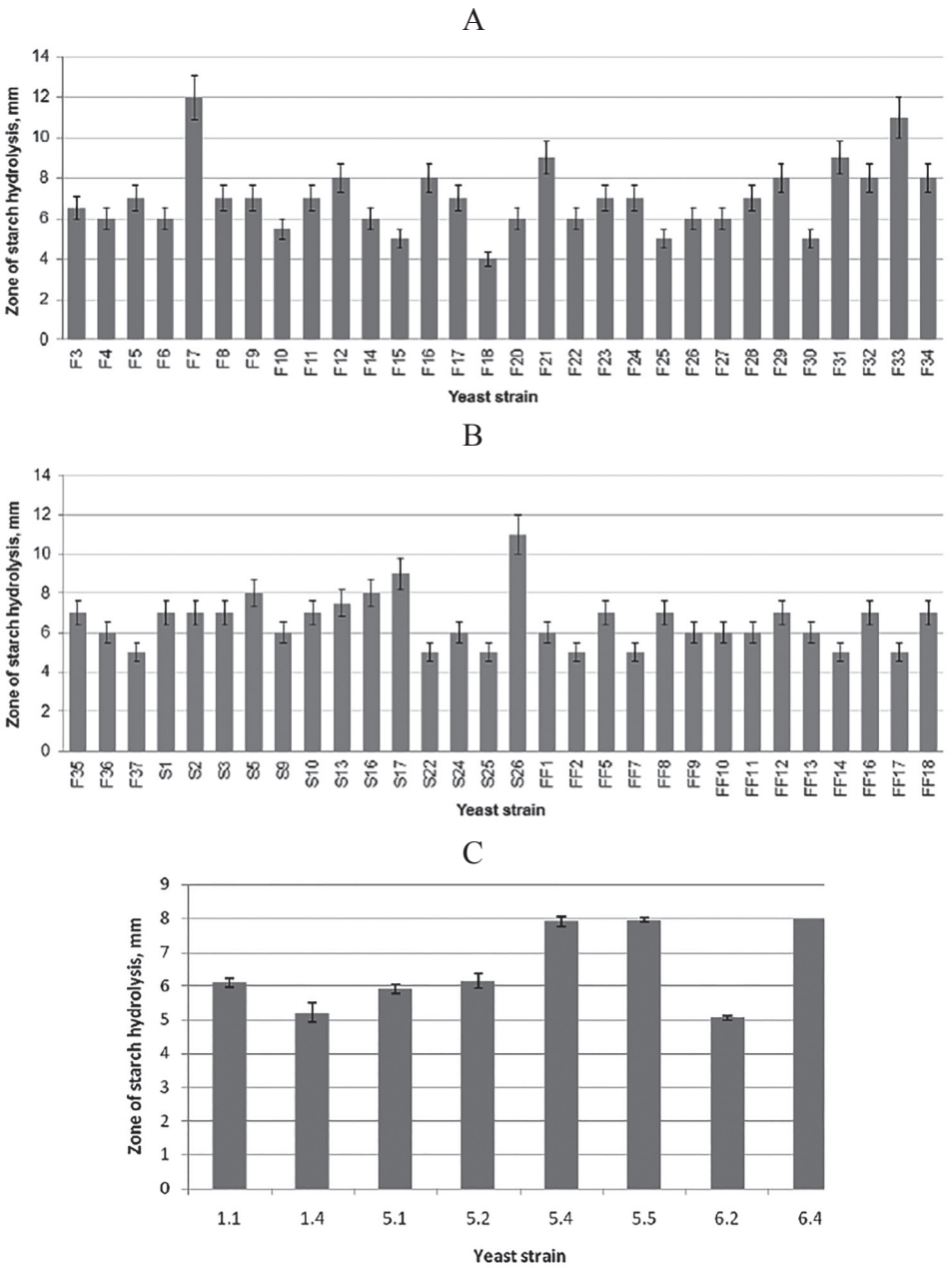

Figure 1. Zone of starch hydrolysis formed by yeasts isolated from dairy products (A, B) and crops (C)

Six strains with the largest zone of starch hydrolysis selected for further research were identified as *Torulaspora* sp. F7 (isolated from sour cream) and *Shwanniomyces vanrijiae var. yarowii F33* and *Candida* sp. S26 (isolated from cheese) and *Lipomyces mesembrius* 5.4, 5.5 and 6.4 (isolated from apples).

It was reported that few yeasts belonging to *Saccharomyces diastaticus* and *Endomycopsis capsularis* besides amylolytic activity were also capable

to produce ethanol from starch [14]. Such yeasts converted starch to ethanol with comparatively low efficiency of 21–38 %. As a result the use of these strains for direct conversion of starch into ethanol is not economically efficient and ethanol yield by saccharomycetes is much higher. The co-cultivation of saccharomycetes together with amylolytic yeasts might be more promising for bioethanol production [2, 14].

The ability of the selected strains to assimilate starch and produce ethanol was studied at 28–30 °C under aerobic (207 rpm at the rotary shaker) and stationary conditions (incubator without agitation) (Fig. 2, A–F).

Strain *Lipomyces mesembrius sp* 5.4 assimilated 37–45 % of starch present in the medium after 6 days of cultivation (Fig. 2A, Table. 2). The highest biomass production was observed after 4 days of cultivation under aerobic

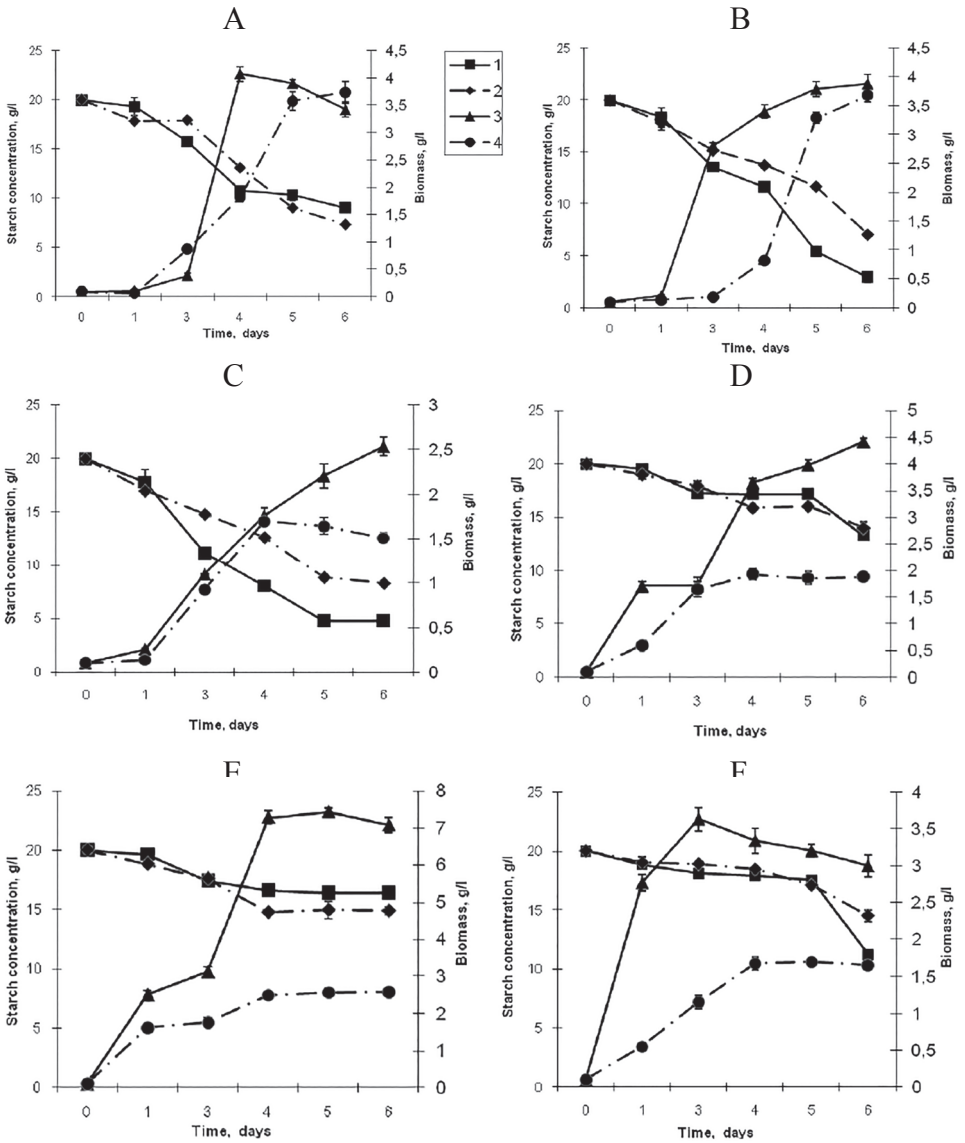

Figure 2. Growth (3–4) and starch assimilation (1–2) by the selected yeast strains: *Lipomyces mesembrius sp* 5.4 (A), 5.5 (B), 6.4 (C); *Torulaspora* sp. F7 (D); *Shwanniomyces vanrijiae var. yarowii F33* (E) and *Candida* sp. S26 (F) under aerobic (solid line) and stationary (dotted line) conditions

conditions (4 g/l) while under stationary conditions the yeast growth was considerably slower. Ethanol concentration after 4 days of cultivation reached 0.044–0.094 g/l, or 0.8–2.3 % of theoretical yield. Strain *Lipomyces mesembrius* 5.5 utilized nearly 85 % of starch under aerobic conditions and 65 % of starch under stationary conditions (Fig. 2B, Table 3). Yeast growth under aerobic conditions was also more pronounced. Ethanol production from starch was very low and comprised 0.57–2 % of theoretical yield. Strain *Lipomyces mesembrius* 6.4 assimilated 76 % of starch under aerobic conditions and 59 % of starch under stationary conditions (Fig. 3C, Table 3). Biomass production during first 4 days of cultivation did not greatly differ under aerobic and stationary conditions. Ethanol production was almost negligible as by other *Lypomyces* isolates and comprised 0.53–3 % of theoretical yield.

Strain *Torulaspora* sp. F7 assimilated 30–33 % of starch after 6 days of cultivation (Fig. 2D, Table. 3). Ethanol concentration after 96 h of cultivation reached 0.014–0.066 g/l, or 0.87–2.83 % of theoretical yield. Strain *Shwanniomyces vanrijiae var. yarowii F33* utilized 18 % of starch under aerobic and 25 % of starch under stationary conditions after 6 days of cultivation (Fig. 2E, Table 3).

Table 3

Ethanol production from starch under aerobic and stationary conditions

Yeast strain	Cultivation conditions			
	Aerobic		Stationary	
	$Y_{P/S}$ g ethanol / g starch	$Y_{\%T}$ (% of theoretical yield)	$Y_{P/S}$ g ethanol / g starch	$Y_{\%T}$ (% of theoretical yield)
5.4	0.0047	0.839	0.013	2.32
5.5	0.0032	0.57	0.0113	2.02
6.4	0.003	0.535	0.017	3.03
F7	0.0049	0.875	0.0159	2.83
F33	0.0017	0.303	0.00076	0.135
S26	0.0038	0.67	0.0058	1.03

Table 4

Ethanol production under microaerobic conditions

Yeast strain	Ethanol concentration g/l	$Y_{P/S}$ g ethanol / g starch	$Y_{\%T}$ (% of theoretical yield)
L. mesembrius 5.4	0.184	0.025	4.46
L. mesembrius 5.5	0.089	0.009	1.61
L. mesembrius 6.4	0.356	0.028	5.0
Torulaspora sp. F7	0.191	0.03	5.35
S. vanrijiae var. yarowii F33	0.211	0.034	6.07
Candida sp. S26	0	–	–

Ethanol production comprised only 0.13–0.3 % of theoretical yield. Strain *Candida* sp. S26 assimilated 44 % of starch under aerobic conditions and 27 % of starch under stationary conditions (Fig. 2F, Table 3). Ethanol synthesis was 0.67–1.03 % theoretical yield.

Under microaerobic conditions ethanol production by the selected strains increased to some extent but still remained very low being 1.61–6.07 % of theoretical yield (Table 4). The most active ethanol producers were strains

L. mesembrius 6.4 and *S. vanrijiae var. yarowii F33*. Strain S26 did not produce ethanol under such conditions.

In conclusion, the selected yeast strains were capable of starch utilization under aerobic and stationary conditions; however ethanol production from starch was negligible. These amylolytic yeast strains will be studied as the potential candidates for the further cocultivation with efficient ethanol producers which do not possess the ability to directly hydrolyze starch (e.g. yeasts belonging to genera *Saccharomyces* or *Kluyveromyces*) thus providing a solution for starch-based bioethanol production.

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В.С. Підгорський, О.Д. Янєва, М.О. Фоміна, К.С. Ткаченко, В.С. Стоян

Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України,

вул. Академіка Заболотного, 154, Київ, 03143, Україна

ПРОДУКУВАННЯ ЕТАНОЛУ З КРОХМАЛЮ ДРІЖДЖАМИ, ІЗОЛЬОВАНИМИ З СІЛЬСЬКОГОСПОДАРСЬКИХ КУЛЬТУР ТА МОЛОЧНИХ ПРОДУКТІВ

Резюме

Метою цієї роботи було дослідити здатність дріжджів, ізольованих з сільськогосподарських культур та молочних продуктів, продукувати етанол з крохмалю.

Був проведений скринінг амілолітичних дріжджів серед ізольованих дріжджів. Шість найбільш активних амілолітичних штамів були ідентифіковані як *Lipomyces mesembrius* spp. 5.4, 5.5 та 6.4, *Shwanniomyces vanrijiae var. yarowii* F33, *Torulaspora* sp. F7 та *Candida* sp. S26. З крохмалю відібрані дріжджі продукували етанол в низькій концентрації за аеробних умов – 0,006–0,129 g/l (0,3–0,87 % теоретично можливого виходу) та мікроаеробних умов – 0,089–0,35 г/л (1,61–6,07 % теоретично можливого виходу). Такі штами будуть досліджені як потенційні кандидати для сумісного культивування з ефективними продуцентами етанолу, що не мають амілолітичної активності.

Ключові слова: амілолітичні дріжджі, етанол, крохмаль, біопаливо.

В.С. Подгорский, О.Д. Янева, М.А. Фомина, Е.С. Ткаченко, В.С. Стоян

Институт микробиологии и вирусологии НАН Украины,
ул. Академика Заболотного, 154, Киев, 03143, Украина

ПОЛУЧЕНИЕ ЭТАНОЛА ИЗ КРАХМАЛА ДРОЖЖАМИ, ВЫДЕЛЕННЫМИ ИЗ СЕЛЬСКОХОЗЯЙСТВЕННЫХ КУЛЬТУР И МОЛОЧНЫХ ПРОДУКТОВ

Резюме

Целью данной работы было изучить способность дрожжей, выделенных из сельскохозяйственных культур и молочных продуктов, конвертировать крахмал в этанол.

Был проведен скрининг амилолитических дрожжей среди изолированных дрожжей. Шесть наиболее активных штаммов были идентифицированы как *Lipomyces*

mesembrius spp. 5.4, 5.5 и 6.4, *Shwanniomyces vanrijiae var. yarowii* F33, *Torulaspora* sp. F7 и *Candida* sp. S26. Из крахмала отобранные дрожжи продуцировали этанол в низкой концентрации в аэробных условиях – 0,006–0,129 g/l (0,3–0,87 % теоретически возможного) и микроаэробных условиях – 0,089–0,35 г/л (1,61–6,07 % теоретически возможного). Такие штаммы будут изучены в качестве потенциальных кандидатов для совместного культивирования с эффективными продуцентами этанола, не обладающими амилолитической активностью.

К л ю ч е в ы е с л о в а: амилолитические дрожжи, этанол, крахмал, биотопливо.

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