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OBTAINING YEAST CULTURES FOR ALCOHOLIC FERMENTATION FROM LYOPHILIZED STRAINS *SACCHAROMYCES*

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Abstract

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The objective of the present study is to obtain yeast cultures for alcoholic fermentation from lyophilized strains *Saccharomyces* with high number of viable cells. Two types of strains of brewer's yeasts – the strain *Saccaharomyces uvarium carlbergensis* No 569 and the strain *Saccharomyces cerevisiae* No 576 are used, which are included in the Catalogue for National Bank for Industrial Microorganisms and Cell Cultures. According to their certificate, they are used for fermentation of sugars of the wheat grain and as a result ethyl alcohol up to 8 $g.kg^{-1}$ is obtained. Two methods have been applied for yeasts cultivation during the process of obtaining yeast cultures: deep cultivation in a liquid medium and surface cultivation on agar medium. As a result of many experiments for cultivation of yeast strains, it was determined the highest growth for colonies can be achieved on Sabury medium with 4 $g.kg^{-1}$ dextrose at 30°C, pH 6.0 – 6.2 and time of cultivation time - 72 hours. In the laboratory cultures, obtained from both tested strains, we found high number of viable yeast cells and the values obtained in strain No 576 were 10^8 colony-forming (CFU) units/cm^3.The culture obtained was an industrial monoculture, containing 9,66. 10^8 CFU/cm^3 living yeast cells of strain 576 which exceeded the results obtained by other scientists. This method is considered as a new one for the practice and may provide intensive process for alcoholic fermentation. We investigated the factors having influence on the growth and development of the yeasts cells during the process of obtaining yeast cultures and their parameters for optimizing the fermentation process. The results obtained can serve as a proof for fermentation and reproductive activity of the selected strains of brewer's yeasts. In that way, the wheat grain may be used as a valuable source for the production of bioethanol through alcoholic fermentation.

Key words: brewer's yeasts, yeast cultures, number of viable cells, fermentation.
Abbreviations: NBIMCC - National Bank for Industrial Microorganisms and Cell Cultures
CFU/cm^3 – colony-forming units/1 cm^3

Introduction

Alcoholic fermentation is a complicated biochemical process carried out by yeasts and it also implies the conversion of sugars, particularly monosugars into ethyl alcohol and carbon dioxide.

Main sources of alcoholic fermentation are various grain products, molasses, sulphite lye and wood

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residues and in the last years potatoes as well as rapes have been also experimented. Cereal crops such as wheat, maize, barley, rye malt are also used for fermentation.

Typical yeasts for fermentation belong to the family Saccharomycetaceae. In the practice more often strains of *Saccharomyces cerevisiae* and rarely *S. ellipsodeius* are used. Strains-producers should reproduce themselves quickly, to be resistant to high concentrations of alcohol and sugars, have high fermentation activity and not many residual products left. (Ginova-Stoyanova and Mileva, 2001; Dyakonov and Marinov, 2001).

Optimal pH-value for the growth of more types of yeasts is in the range from 3.5 to 7.5, and for the industrial fermentation the values vary from 4.8 to 5.0 (Raduchev and Rizvanov, 1971; Beshkov, 1974).

Alcoholic fermentation is usually performed at optimal temperature about 30^0C, but not higher than 32^0C, accompanied by shaking for 40-60 hours, after that the fermentation mixture contains 6.5 – 8.5 volume $g.kg^{-1}$ alcohol (Beshkov, 1974).

The preparation of the yeasts for alcoholic fermentation is of great importance for the overall process, which includes two stages: laboratory industrial. The condition of the yeast cultures determines to a great extent the technological process and qualitative indicators of the alcohol obtained (Kirov et al., 1971; Ruzhich, 2000; Bambalov et al., 2000). When pure strains are used, it is necessary to be prepared such monoculture which has to contain a certain number of viable yeast cells. According to Ivanov et al. (1979), the number of yeast cells should be 5.6 million/ cm^3, while according to Ruzhich (2000) this quantity should be 3 million/1 cm^3.

During brewery production, Popova et al. (2001) used yeast cultures containing 20-30 million/cm^3 yeast cells. Ginova- Stoyanova and Mileva (2001) recomend the number of viable cells to be above 90 $g.kg^{-1}$ at maximum number of cells not less than 45-50 million/ cm^3. Lihtenberg (1977) tested yeast culture containing 98.5 million/cm^3 at 2 $g.kg^{-1}$ dead cells. Beshkov (1974) suggests a method for obtaining yeast culture in which after incubation is achieved a concentration of yeast cells about 400 -500 million/cm^3.

Taking into account the fact that some authors indicate different number of yeast cells, it becomes necessary to optimize the existing methods for cultivation of selected brewer's yeast strains in order to obtain industrial cultures with maximum number of viable cells and their usage for alcoholic fermentation.

On the other hand, the variety of strains requires the selection of such types which should have high reproductive and fermentation activity and will conduct the conversion sugars of wheat into ethyl alcohol. Wheat grain might be a source for the production of bioethanol through alcoholic fermentation.

Objectives

The objectives of the study were to obtain high-quality yeast laboratory and industrial cultures with specific characteristics of the selected lyophilized yeast strains with high concentration of viable cells and to be used for fermentation of sugars from the wheat grain and production of ethyl alcohol.

In order to achieve the above-mentioned objectives, the following tasks were outlined:

- Activation and cultivation of two yeast strains as to obtain yeast cultures with maximum number of viable cells;
- Determination of reproductive activity of the selected yeast strains;
- Optimization of the parameters for obtaining yeast cultures and speeding the fermentation process.

Materials and Methods

The materials used were two types of brewer's yeasts: *Saccahromyces* No 569 and No 576, from the Catalogue of NBIMCC. The strain specificity of these strains was as followed:

• Strain No 569 - *Saccharomyces uvarium carlbergensis*

• Strain No 567 - *Saccharomyces cerevisiae*.

The strains were obtained in NBIMCC in compliance with the method of vacuum drying and then stored in ampoules, called cryotubes. According to the information in the Catalogue, these brewer's yeasts are used for fermentation of sugars from the wheat, and as a result ethyl alcohol up to 8 g.kg $^{-1}$ is obtained.

Methods

• Rehydration, activation and restoration of the lyophilized strains;

• Cultivation of the strains as separate and mixed cultures – two methods for yeast cultivation are applied: deep cultivation in liquid medium and surface cultivation on agar medium;

• Indirect method for determination of the number of yeast cells in suspension;

• Direct method for determination the number of dead cells – according to Ivanov et al. (1979).

Results and Discussion

The investigations conducted and results obtained can be presented as follows:

• Preparation of strains –rehydration, activation and restoration.

The content from the ampoule was poured into test tubes with 1.8 cm^3 physiological solution. In order to activate the yeast cells, the test tube was put in a thermostat at 30^{0}C for 30 minutes – time sufficient to restore their viability. The restored strain from the suspension obtained was cultivated on different media in compliance with above mentioned methods. For the yeast cultivation we used liquid and agar nutritive media. The liquid medium contains yeast extract – bouillon, and agar medium - yeast extract – agar, Sabury agar with 4 g.kg $^{-1}$ dextrose and beer agar.

We traced the influence of various factors on the growth and development of yeast cells such as composition of the nutritive medium, pH value of the medium, method of cultivation, temperature regime.

When applying the deep method, the suspension of the restored strain was distributed in different quantities from fluid agar medium /50, 100, 150 cm^3/. The reproduction of yeast cells was conducted statically in a thermostat or mechanically through shaking. The samples were cultivated in a thermostat at 30^{0}C for 48 -72 hours. In case of intense growth, the yeast cells biomass remains in the bottom of the flask in the form of white sediment.

When applying the surface method, the restored strain from the suspension was grown on the agar surface. The flasks were cultivated in a thermostat at 30^{0}C for 48- 72 hours.

As a result of the experiments conducted and previous numerous investigations for yeast cultivation on different nutritive media, it was found that intensive growth of colonies can be observed only in case of applying the surface method on Sabury agar – 4 g. kg-^{1} dextrose, pH-value of the medium 6.0 – 6.2, at 30^{0}C and time for cultivation 72 hours. In that way one of the objectives was achieved.

When applying the surface yeast cultivation in liquid nutritive medium, the growth of the bacteria cells was not so intensive. Therefore during our further investigations we continued to apply the surface method of yeast cultivation.

• Preparation of laboratory cultures.

According to the above mentioned method, the biomass with the help of a sterile loop was inoculated again on Sabury agar. The yeasts were cultivated on Sabury agar at 28-30^o C for 48-72 hours.

Determination of the number of viable cells of the selected strains was conducted in the following way:

Sterile physiological solution (2 cm^3) was added to the content of each flask with cultivated yeasts. As a result of energetic shaking, suspension – 10^0 was obtained. Dilutions from 10^{-1} to 10^{-9} from the suspension were prepared and grown on Sabury nutritive

Table 1
Average values for the number of yeast cells (CFU/cm^3) in laboratory monocultures in separate experiments

№ of experiment	Number of living cells CFU/cm^3	
	Monocultures of strain 576	Monocultures of strain 569
1.	$9.66.10^8$	$1.86.10^8$
2.	$6.27.10^7$	$3.45.10^7$
3.	$1.50.10^7$	$4.10.10^7$
4.	$3.10.10^7$	$2.90.10^7$
5.	$4.60.10^8$	$5.60.10^7$
6.	$6.51.10^8$	$1.08.10^8$
7.	$8.26.10^8$	$1.24.10^8$

Table 2
Concentration of living yeast cells in industrial cultures during cultivation

Period of cultivation / Type of culture	Concentration of living yeast cells CFU/cm^3			
	6 h	24 h	48 h	72 h
1. Monoculture of strain 569	$3.1.10^5$	$2.9.10^6$	$5.6.10^7$	$1.86.10^8$
2. Monoculture of strain 576	$6.4.10^5$	$1.5.10^7$	$6.27.10^7$	$9.66.10^8$
3. Mixed culture of both strains 569 and 576 (1 : 1)	$5.33.10^4$	$4.7.10^5$	$8.25.10^6$	$3.5.10^7$

agar medium. Then yeasts were cultivated in a thermostat at 28-30^{0}C for 72 hours. The colonies obtained were directly counted and the number of each separate dilution and parallel to each sample the average values were measured.

In Table 1 are presented the average values of the number of colony-forming units/cm^3 in laboratory monocultures from 7 experiments, with 4 consecutive dilutions and 3 repetitions.

Data (Table 1) show, the laboratory monocultures of both strains have high number of viable cells, obtained by the surface method and cultivated under the above-described conditions. It should be noted, the number of viable cells of strain No 576 is higher (10^8 CFU/cm^3) during most experiments conducted.

It was carried out indirect determination of the number of yeast cells in monocultures, obtained in the case of the deep cultivation method, but the results ware lower than those of the surface method – from 1.5 to $5.6.10^7$ CFU/cm^3.

• Preparation of industrial cultures

Many experiments were conducted in which yeast biomass cells from the agar was mixed with yeast extract bouillon in different proportions.

Best results were obtained in the following case: to the yeast biomass in two test tubes was added yeast extract bouillon and the mixture shaken. Then this mixture with the yeast biomass was poured into a sterile flask. The remaining biomass was shaken several times with the same nutritive media and poured into the same sterile flask but the total quantity of bouillon should be 100 cm^3. Yeast cultivation in the suspension obtained is conducted in a thermostat at 28 - 30^{0}C for 72 hours. During cultivation we took samples to determine the number of yeast cells at the 6-th, 24-th, 48-th and 72-th hour.

During the experiments we used separate monocultures of both selected strains as well as combination between them in proportion 1:1. The combination was obtained by mixture of strain No 576 from one test-tube and mixture of strain No 596. The total quantity of the yeast bouillon was again 100 cm^3.

Results from the yeast concentration of yeast cells in the industrial cultures during cultivation are presented in Table 2.

It is evident from Table 2, the concentration of viable cells in both yeast strains at the 72-th hour after cultivation was varying from 1.86 to $9.66.10^8$ CFU/cm^3. The results obtained for the number of viable cells may serve as a proof for high- quality yeast culture for industrial fermentation. The highest value was

measured in the monoculture of the strain No 576- $9.66.10^8$ or 966 million CFU/cm^3. In the mixed culture the concentration of viable cells was lower in comparison with the monocultures but may be also used for alcoholic fermentation.

The concentration of viable yeast cells in the industrial yeast cultures, obtained by us, is higher than that determined by other authors. (Beshkov, 1974; Lihtenberg, 1977; Ivanov et al., 1979; Ginova-Stoyanova and Mileva, 2001; Popova et al., 2001). This is new for the practice and can provide intensive process for alcoholic fermentation and respectively better production of ethyl alcohol.

In order to determine the viable condition of yeast cells in the industrial cultures, we applied a direct method for counting the dead cells (Ivanov et al., 1979). According to the method, on a piece of glass a drop of the strain and a drop from the colour substance – rhodamine were dripped, then covered by a glass and microscoped. Dead cells become red while the viable cells remained uncoloured. Then the number of the coloured and uncoloured cells is counted and calculated the average number of viable cells. This method is considered as a subjective one, and therefore it does not guarantee certain results.

Our investigations, referring to the number of viable and dead cells, at 72-the hour during cultivation, indicate the average values from the three experiments with three repetitions:

- Monoculture of strain No 576 contained average 99 $g.kg^{-1}$ viable cells.
- Monoculture of strain No 569 contained average 95 $g.kg^{-1}$ viable cells.
- Mixed bacterial culture from both strains contained average 90 $g.kg^{-1}$ viable cells.

According to Ivanov et al. (1979), the quantity of dead cells should be from 5 to 10 $g.kg^{-1}$ and such results were also proved by our experiments.

The above described method for producing industrial yeast cultures may contribute to the practice for obtaining industrial yeast cultures for alcoholic fermentation.

The results presented for the concentration of viable yeast cells may serve as a proof that we achieved high reproductive and fermentation activity of the selected brewer's yeasts which are used for fermentation from sugars of the wheat. When the semi-industrial fermentor "Bio-engineering" was loaded with industrial yeast cultures as a result a distillate with high content of alcohol (70^0 ethanol) was obtained. So, in the future, high quality yeast cultures of the wheat grain may be used as a valuable source for bioethanol production through alcoholic fermentation.

Conclusions

The conditions for restoration, cultivation and obtaining laboratory cultures of both strains lyophilized brewer's yeasts are optimized.

In the laboratory cultures is determined a high number of viable yeast cells, higher in strain No 576 - 10^8 CFU/cm^3.

An industrial monoculture is obtained which contains $9.66.10^8$ CFU/cm^3 viable cells of strain No 576. The monoculture obtained may be used as a high quality yeast culture for alcoholic fermentation.

The developed method for obtaining industrial yeast cultures contributes to the practice through intensification of the process for alcoholic fermentation.

The factors, having influence on the growth of yeast cells, are analyzed and their optimal parameters determined, helping for speeding the fermentation process.

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