JAP&T TPA-Timişoara

Available on-line at www.japt.tpa.usab-tm.ro

Journal of Agroalimentary Processes and Technologies 2009, *15*(1), 107-111

Journal of Agroalimentary Processes and Technologies

Isolation and selection of high ethanol producing yeast strains

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Abstract

There were isolated 41 yeast strains, for isolation being used both natural and special prepared laboratory media. 10 yeast strains were studied as regards their capacity to produce high ethanol concentration, to ferment different raw materials and to be resistant to high ethanol concentration, in order to be used to produce bioethanol from starchy raw-material (mainly corn). This selection was performed based on the fermentation properties specific for every yeast strain and the main parameter was the ethanol content in fermented mash.

For every yeast strain tested in pilot installation three successive repetitions were performed in the case of molasses as raw material and five successive repetitions were performed in the case of corn as raw material. The working conditions at laboratory and pilot levels were similar as regards the technology and equipment.

Keywords: Alcoholic fermentation, bioethanol, cereals, selection, pilot tests.

1. Introduction

Yeasts are mainly the micro-organisms able to perform the alcoholic fermentation, meaning the transformation of sugar into ethanol and carbon dioxide, without involvement of free oxygen. The alcoholic fermentation is performed by different yeast, but mainly by *Saccharomyces* species, but can be also performed by some moulds (such as *Mucor*, *Aspergillius* or *Penicillium*) and specific bacteria. The utilization of yeasts in fermentation industries or biotechnologies involves pure cultures [1, 2, 3].

For the identification of a new isolated yeast strain it is necessary to carry on a precise characterization and description that allow the comparison with already known yeast. The main features used for the yeast identification are the morphological characteristics of vegetative cells, the multiplication the biochemical and physiological characteristics [2].

The selection of micro-organisms to produce ethanol takes into account the specific features of the technological process and the available equipment. The micro-organisms used for the alcoholic fermentation have to meet the requirements of the specific fermentation process and also of the requirements of ethanol producer.

The main requirements refer to the next topics:

- high yield in ethanol related to the unit of substrate;
- high fermentation capacity;
- tolerance for high ethanol concentration;
- tolerance for small pH values;
- small requirements for oxygen;
- high stability under different fermentation conditions;
- utilization of a large range of fermentable substrates.

Yeast belonging to *Saccharomyces* genus with the different species (*cerevisiae*, *carlsbergensis*, *ellipsoideus*, *oviformis*) and varieties, are used in fermentation industries such as ethanol, baking, wine and brewing industry. Through mutation and

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selections the wild yeasts were adapted to the specific industrial conditions, loosing their initial characteristics and developing new and important characteristics for the fermentation industries [2].

The selection of yeasts able to ferment under optimal conditions the media containing starchy or sugar as fermentable substrate has to study both the quantitative and qualitative aspect of ethanol production through fermentation.
The alcoholic forming power represents the high ethanol concentration which can be accumulated when within fermentation medium there is an excess of sugar. Generally speaking the yeasts are sensitive to ethanol, but in comparison with *Kloeckera* and *Torulopsis* genera, that are inhibited at ethanol concentration at about 4-6%v/v, the (*Saccharomyces* yeasts *Saccharomyces cerevisiae* var. *ellipsoideus*, *Saccharomyces cerevisiae* - *cerevisiae*) have a high alcoholic forming power and are able to continue the fermentation until the accumulation of 16-18% v/v ethanol in culture medium.

The alcohol resistance represents the capacity of yeast to start the fermentation in presence of 8% v/v ethanol and to continue the fermentation until high ethanol concentration [3].

An appropriate high ethanol producing yeast has to possess both characteristics – a high alcoholic forming power and a high alcohol resistance

The present paper presents aspects related to isolation and selection of yeasts able to produce through fermentation high ethanol concentration in order to be used in industrial equipments to produce bioethanol from renewable agricultural raw-materials such as grains (corn) and molasses.

2. Materials and Method

Saccharomyces cerevisiae strains used to produce bioethanol have to meet the following main requests:

- to be stable from genetically point of view and to maintain the fermentation performances for many fermentation repetition;
- to be able to perform the fermentation of the medium as soon as possible and to produce ethanol with a yield as close as possible to the theoretical yield;
- to be not very exigent as regard the growing factors in order to limit the vitamins supplement in the case of industrial fermentation medium.

The most used isolation method of yeasts in pure culture is the combined method Lindner – Koch. The method based on cells spread in nutritive liquid media and at distance fixation of the colonies after medium solidification, isolated colonies each other forming through multiplication [1].

During the study natural media were used and the following steps were covered:

- yeasts isolation as pure culture;
- macroscopical and microscopical characterisation of yeasts;
- physiological and cultural characterisation and study of fermentation behaviour.
- The directions tackled during the study were the following:
- laboratory experiments in order to select and test the yeast strains able to carry on high fermentation yield;
- pilot experiments in order to verify the alcoholic forming power of isolated yeasts and optimal fermentation technology.

There were isolated in laboratory 41 yeast strains, for isolation being used both natural and special prepared laboratory media.

Isolation was performed from fruits and their juices and from the air, using special media and different mashes in pilot plant (sugar, molasses and grain mashes) and also their dilutions, using classical methods of spreading and scarifying. a medium based on molasses and another based on corn as special media were used [4].

The selection medium used during experiments has the following formula: malt extract 0.3%, glucose 1%, yeast

extract 0.3%, peptone 0.5% and agar-agar 2%.

The medium is distributed in conical vessels and after sterilisation and cooling the inoculation is performed using an active cells suspension (10^6 cells/ml). The fermentation valves are settled and the samples are weighted. The samples are incubated 48 hours at 280C and after this period they are weighted again. The strains having the best fermentation capacity and speed are selected.

The medium used for re-pitching and conservation is malt wort with agar (malt wort 8% + 2% agar).

After colonies growth the pure cultures were preliminary studied from morpho-physiological point of view using both solid and liquid media.

The identification of selected yeast strains (for genera and species) was performed using the system API ID32C produced by the company Biomerieux (France). The system ID32C is a standardised system used to identify yeasts. The system has 32 miniaturized assimilation tests and a data base and the reading of results can be performed manual or automatic. Only pure cultures of a single organism should be used.

The ID32C strip consists of 32 cupules, each containing a dehydrated carbohydrate substrate. A semi-solid, minimal medium is inoculated with a suspension of yeast organism to be tested. After 48 hours of incubation at 290C ±20C, growth in each cupule is read visually comparing each cupule to the control (0) and record as positive any cupule that is more turbid.

Interpretation is obtained using the database. The results are introduced into the apiwebTM identification software manually [5].

The verification of yeast strains on corn mashes was performed taking into account the industrial technological process. After fermentation of mashes during 72 hours at 30 - 320C the alcohol content through distillation was performed.

The pilot experiments were performed both to verify the behaviour of selected yeasts strains during alcoholic fermentation of different agricultural raw-materials and also to adapt the strains to high gravity and very high gravity mashes and then to adapt to similar industrial conditions used at present in bioethanol plants.

The control strain was a baking yeasts (Saccharomyces cerevisiae) that is normally used at present in Romanian ethanol plants. The indicative of this strain is DP.

For every yeast strain tested in pilot installation three successive repetitions were performed in the case of molasses as raw material and five successive repetitions were performed in the case of corn as raw material. The working conditions at laboratory and pilot levels were similar as regards the technology and equipment.

During the experiments the evolution of ethanol content, total sugars and fermentable sugars in mashes were performed, in order to settle the fermentation performances [6, 4].

3. Results and Discussion

After isolation of 41 yeasts strains from natural and special prepared laboratory media, their were tested and 10 strains were selected and studied as regards their capacity to produce high ethanol concentration in order to be used to produce bioethanol from starchy raw-material (mainly corn) and from molasses. This selection was performed based on the fermentation properties specific for every yeast strain and the main parameter was the ethanol content in fermented mash.

The morpho-physiological testing of isolated and selected yeasts comprises the following steps:

- determination of the culture characteristics in solid media using solid YPG medium;
- determination of the culture characteristics in liquid media using malt wort 8%;

- microscopical analysis of both liquid and solid media were performed.
- determination of fermentation capacity for the main sugars using the auxonographic method in liquid and solid medium. The selected sugars were glucose, saccharose, maltose, fructose, gallactose and raffinose.

The samples in fermentation were also studied for the speed of fermentation through daily weighing (during the fermentation duration - 72 hours), after the removing of carbon dioxide.

All 10 yeast strains registered a high and fast adaptation capacity and fermentation capacity releasing high carbon dioxide quantities after 48 hours of fermentation.

- utilisation of ethanol as unique carbon source using a nutritive medium containing ethanol (3%), (NH4)2SO4 (0.1%) , KH2PO4 (0.1%), MgSO4 x 7H2O (0.01%), distilled water.

After repartition in tubes, the culture medium is sterilised at 1 bar for 15 minutes and inoculated after cooling with an active yeasts cells suspension and is incubated 3 days at 280C. The test is positive when the cells growth and a deposit are observed. The tests were performed in comparison with a control test without ethanol.

The test was positive for all 10 selected strains.

- morphology of vegetative cells and their multiplication characteristics;
- capacity to form filaments and particularly morphological characteristics through [1, 2].

The results for API ID32C for the 10 strains isolated, selected and tested in laboratory and in pilot plant, demonstrated that all belong to Saccharomyces genus, species cerevisiae.

The pilot tests were performed with the yeasts strains having the best performances at laboratory tests. The paper presents the results of the fermentation experiments performed only for corn mashes, but also molasses mashes were analyzed, with similar results.

In the case of corn mashes 5 repetitions were performed for every yeast strain, working in the same technological conditions – working parameters and equipment.

During pilot tests the fermentation performances were evaluated, measuring the next main indicators:

- ethanol content – by distillation method (using the pycnometer);
- total sugar content using the Luff-Schoorl method;
- fermentable sugar content (glucose content) using the Luff-Schoorl method.

These key parameters were measured every 2 hours during the 72 hours fermentation duration.

The pilot experiments were performed in 20 litres fermentation vessels endowed with automatic control of temperature.

The corn mashes were liquefied and saccharificated using NOVOZYMES industrial enzymes. The enzymes used within the tests were the following:

- Liquozyme SC DS for liquefaction;
- Spirizyme Fuel for saccharification.

Spirizyme Fuel enzyme was tested in system of simultaneous saccharification and fermentation (SSF) as well as for short saccharification (2-hour short saccharification followed by fermentation).

The ratio corn : water was 1 : 2 and the natural pH of the mash was 5.85.

The technological variant was with pH 4.5 through H2SO4 addition and the maintenance at this value during test performance through addition of solution 25% NH3 [6, 7].

The quality parameters of corn used in experiments were the following:

- moisture – 11.55%;
- starch – 63.28% d.m.

The fermentation parameters of the corn mash were the following:

- temperature – 300C;
- pH – 4.5 ÷ 4.8.

The results obtained for the alcohol content of fermented mashes using the isolated yeasts strains are presented in figure 1. The figure presents the medium value of ethanol content obtained for the 5 fermentations of corn mashes for every yeast strain.

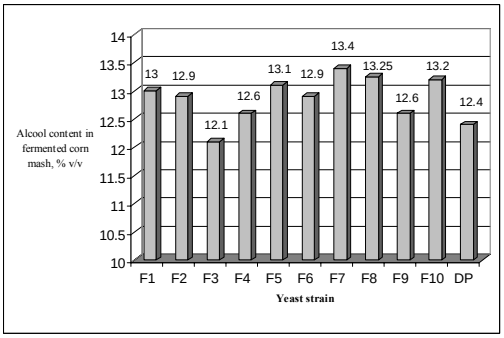

Figure 1: Alcohol content in fermented corn mashes

The results at the end of fermentation process using F7 strain are presented in table 1.

Table 1. Fermentation performances of F7 Saccharomyces cerevisiae strain

Test no.	Alcohol content in fermented corn mashes, % v/v	Total residual sugar, %	Glucose, %
1	12.05	1.23	1.03
2	13.56	1.08	0.07
3	13.59	1.12	0.06
4	14.11	0.98	0.05
5	13.72	1.12	0.07

Based on the fermentation performances F7 strain registered the best results at tests performed at laboratory and pilot level.

Through the tests performed in pilot plant the strains F7 was noticed as the best one, producing the highest ethanol concentration for corn mashes, but also for molasses mashes (the results for the fermentation of molasses were not presented in this paper).

4. Conclusion

- through successive fermentations using substrates with concentration in sugars growing progressively the selection of high ethanol producing yeasts is feasible. These yeasts can be used in order to produce bioethanol at industrial level;
- it is possible to select high ethanol producing yeasts, able to perform the fermentation of different agricultural raw-materials, on the basis of the tests performed at laboratory and pilot level using corn and molasses mashes;
- the utilisation of high ethanol producing yeasts allows to obtain high ethanol yields in combination with the mashing technologies in very high gravity (VHG) and simultaneous saccharification and fermentation (SSF) systems;
- verification of selected yeasts at pilot level has an important contribution in order to adapt the yeasts to high concentration in sugars of the substrate and to stimulate the producing of progressive high ethanol concentration in fermented media.

Acknowledgements

This paper presents the results of the project IDEI 321/2007 „Researches for establishing processing biotechnologies to use renewable indigenous agricultural resources", supported by the Romanian National Plan for Research – Development and Innovation of the Ministry for Education and Research

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