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Characterization of saccharomyces carlsbergensis mutants obtained by nitrous acid mutagenesis

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Abstract

The end of the twentieth century was marked by major advances in life technology, particularly in areas related to genetics and more recently genomics. The genetic improvement of industrial strains traditionally relied on classical genetic techniques (mutagenesis, hybridization, protoplast fusion), followed by selection for broad traits such as fermentation capacity, ethanol tolerance, flocculation or carbohydrate utilization. In this work was tested the alcoholic fermentation potential of Saccharomyces carlsbergensis mutants obtained after nitrous acid treatment. Rates of survival of this mutant strains was calculated and there morphological properties was also characterized.

Keywords: nitrous acid, mutagenesis, Saccharomyces carlsbergensis.

1. Introduction

The brewin yeast Saccharomyces carlsbergensis has been shown to be a hybrid of Saccharomyces cerevisiae and Saccharomyces bayanus (Kiellen-Brandt, 1995; Masneuf, 1998). The strain was isolated by Hansen in 1908. The hybrid nature of this strain may be responsible for its popularity as a brewing strain.

The genome of the organism ultimately controls its metabolism. Although improved fermented engineering design and optimal cultural conditions can quantitatively enhance the microbial products, this will only be up to a limit. Genetic improvement of the organism is fundamental to the success of fermentation technology.

The improvement of the yeast strains was traditionally based on random mutagenesis or classical breeding and genetic crossing of two strains followed by screening for mutants exhibiting enhanced properties of interest. Microorganisms are genetically endowed with a mechanism that fixes the production of metabolic cells to a level that should meet their own needs.

A certain amount of mutational change in the genome occurs as a natural process, though the probability is small. Exposing a culture of a micro-organism to chemicals enhances the rate of occurrence of mutations.

The mutagen capacity of nitrous acid can be explained by it's specifically way of action.

Because of hypoxanthine (similar as guanine) and uracil (similar as timine) properties, in interface of cell cycle, the pairing error of azotate bases, appeared.

The pair A-T is replaced by nucleotide pair C – G and reversed. The transitions G-C A-T and A-T $\Longrightarrow$ G-C induced by nitrous acid determine changes in genetics' information and the appearance of mutations.

But it is a tremendous task for the industrial geneticist to screen the very large number of randomly produced mutants and to select the ones with the desired qualities.

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2. Materials and methods

Yeast strain. The strain *Saccharomyces carlsbergensis* product by S.C. "Nic Prod Trans " SRL Ilfov, Romania.

Obtaining the mutant strains by nitrous acid mutagenesis.

In nature, the nitrous acid is unstable, but in laboratory, it was obtained like a nitrogenous mixture which was realized in 5 tubes:

Solutions	Tubes 1	Tubes 2	Tubes 3	Tubes 4	Tubes 5
$NaNO_2$ 4M	500µl	500µl	500µl	500µl	500µl
NaOH 1%	10 µl	10 µl	10 µl	10 µl	10 µl
Acetic acid 10%	10 µl	20 µl	30 µl	40 µl	50 µl

Solutions	Tub 1'	Tub 2'	Tub 3'	Tub 4'	Tub 5'
Cellular suspension	0,2ml	0,2ml	0,2ml	0,2ml	0,2ml
Nitrogenous mixture	0,2ml	0,2ml	0,2ml	0,2ml	0,2ml
Phosphate-buffer 0,2M pH 7.4	0,4ml	0,4ml	0,4ml	0,4ml	0,4ml

From this tubs with nitrogenous mixture (witch contain different concentration of nitrous acid), it was added about 0,2ml in other tubes in which the mutagenous processes are displayed.

Dilutions are made in phosphate-buffer to 10^{-3}. From the last dilution, of each tub, it was inseminates about 0,1ml on Petri dishes with a YPGA medium followed by luteinizing to obtain isolated colonies. The dishes are incubated at 30°C in the dark for 3 days for invigoration and growth. (Vassu et all., 2000).

Two strains of mutant yeasts taken from the dish witch corresponds to tub 2', 10^{-3} dilution were isolated. The strains were named: *S. carlsbergensis t1* and *S. carlsbergensis t2*, and they were replicated in test-tubes of pure culture of inclined agar malt must and kept in the dark.

Establishing the spectrum of fermented glucides. In each test tube containing 9cm³ Wickerham medium (Table 1) and Durham tube is separately introduced about 1cm sterile solution of galactose, glucose, sucrose, maltose, raffinose. The concentration of the raffinose sterile solution is 40 % and 20% for the other glucides. After the homogenizing process, in each Durham tub is inoculated active *Saccharomyces carlsbergensis* suspension (parental and mutant strains) and it is kept to 28°C, for 10 days. Daily observations are made regarding gas accumulation in the fermentation tubes (Tofan et al., 2002).

Testing the raffinose potential of fermentation.

For the quality determination of the remaining glucides in the medium after testing the raffinose potential of fermentation in the case of the parental strain, the chromatographic gas analyzes is used. For this determination, a 100 µl sample was used. This was made with the help of 0,5ml hexamethyldisylasane (HMDS) and 0,5ml trimethylclorislan (TMCS) in 1ml of dimethylformamide (DMFA).

The reaction time is 24 hours while the two layers are prepared. The derivates that are part of the outer layer are subject to the chromatographic gas analysis. The chromatograph used was VARIAN CP 3800 equipped with an ionization detector in the flame (FID) with the detector temperature of 300°C and an injector with the 1:100 splitting mode at 260°C. The separation was made on a capillary non-pollary column ELITE 5 (Perkin Elmer) with the dimensions of 30m x 0,25mm and a 0, 2µm film. The initial temperature was 140°C, maintained for 3 minutes and then risen to 300°C with a rate of 3°c where it was kept for 20 minutes.

The study of alcoholic fermentation. Obtaining the inoculums. The mutant yeast cells from each test-tube with pure culture, obtained out of inclined agar malt must is transferred in 25cm^3 balloons liquid malt must with a 6°Bllg concentration and is incubated at 26°C for 24 hours for invigoration.

The inoculums obtained is 4% transferred with the help of sterile pipette into 3 fermentation cans that contain 150cm^3 malt must each with 12°Bllg concentration, with fermentation valves. After fixing the fermentation valves, separately sterilized from the can containing the medium, one introduces 2 cm^3 sulphuric acid concentrate through the upper orifice. After weighing each can initially, they are incubated to 30°C for 3 days.

- After 6, 24, 48 and 72 hours the average quantity of CO2 released was calculated.
- Ethanol production was determined by pycnometer method.

3. Results and discussions

After nitrous acid treatment it notices a decrease of cellular viability because of increase concentration at the nitrogenous mixture that was added. In Figure 1 is presented the viability percentage of the *Saccharomyces carlsbergensis* cells, after mutagenesis.

The lethal effect of nitrous acid is due to many causes, for instance-mutations of the genes or chromosomes, destruction of the cell-membrane or cell organelles (Figure 2).

For the morphological characterization of the yeast colonies formed after mutagenesis, two growth mediums were used: *MA*, *YPGA*, whose composition is presented in the *table 1*.

In the table 2 are presented the color, form, profile and diameter of the survival colonies after nitrous acid treatment, for the two growth mediums used. After mutagenesis, the morphological characteristics were almost the same as in the case of untreated cells excepting profile and colonies diameter.

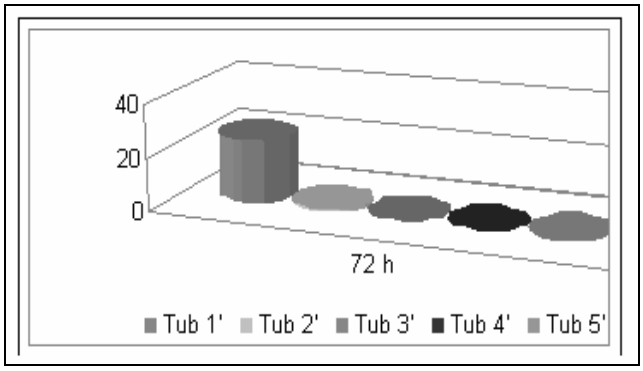

Figure 1. Viability percent of the *Saccharomyces carlsbergensis* cells after nitrous acid mutagenesis

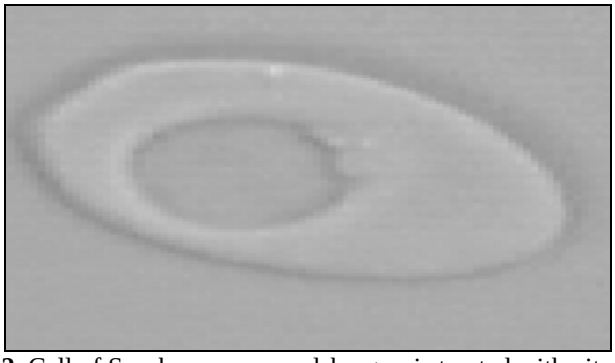

Figure 2. Cell of Saccharomyces carlsbergensis treated with nitrous acid

Table 1. Composition of the cultural mediums

MEDIUMS	COMPOSITION
MA (Malt - agar)	Malt extract 30g; peptone 5g; agar 15 g; distillate water 1L; pH 6,5
YPGA (Yeast-Peptone-Glucose-Agar)	Yeast extract 5g; peptone 10g; glucose 20g; agar 30g; distillate water 1L; pH 7.
Wickerham	Glucose 40g; yeast extract 2,5g; bacto-peptone 5g; $(NH_4)_2SO_4$ 6g; $CaCl_2$. 2 H_2O 0,25g; KH_2PO_4 2g; $MgSO_4.7H_2O$ 0,25g; distillate water 1L; pH 6,5

Table 2. Morphological characterization of the *Saccharomyces carlsbergensis* colonies

Mediums	Characteristics of parental and mutant colonies	
	Parental (10^{-3} dilution)	Mutant (tub 2' , 10^{-3} dilution)
MEA (Malt-extract –agar)	Cream - colored colonies, circular perimeter, lenticular profile, matte and with ± 2,5 mm in diameter;	Cream - colored colonies, circular perimeter, matte and with ± 2,5 mm in diameter;
YPGA (Yeast-Peptone-Glucose-Agar)	Cream-colored colonies , convex profile with circular perimeter, matte and with ± 3,5 mm in diameter;	Cream – colored colonies, convex profile with circular perimeter, matte and with ± 2 mm in diameter;

For the study of alcoholic fermentation, glucides tested were: glucose, galactose, sucrose, maltose and raffinose. The first stage was testing the fermentation potential of glucose both for the parental strain and for the mutant ones. Since the tests proved positive, it was continued the study of other glucides which were, also positive. The test was considered positive because one could notice the release of a sufficient CO2 amount in the Durham tube.

It have been observed that *Saccharomyces carlsbergensis t2* wasn't capable to ferment the maltose comparatively with the wild-type, *S. carlsbergensis* strain and with the mutant strain, *Saccharomyces carlsbergensis t1*, which were capable to ferment all of the glucides.

After the fermentation of the raffinose through the chromatographic gas analysis it was discovered that:

Both in the case of parental yeast, S. carlsbergensis and mutant strain,*S. carlsbergensis t1*, raffinose was entirely fermented. The absence of fructose and glucose in the medium, proves a complete degree of raffinose fermentation (Figure 3).

Alcoholic fermentation is an anaerobic process through which fermentative glucides are metabolized through the oxide-reduction process under the action of the enzymatic equipment of the yeast in the main products (ethanol and CO_2) and secondary products superior alcohol, acids and aldehydes, etc.

The rate of the alcoholic fermentation on the isolated mutant yeast cells compared to the parental cell was made through the estimation of the average CO_2 quantity released in the established period of time.

From the 2 mutant strains whose fermentation capacity was tested, only the *Saccharomyces carlsbergensis t1* released in 72 hours an CO2 quantity similar with the parental *Saccharomyces carlsbergensis* strain (Figure 4).

At fermentation end, the ethanol production was analyzed. The yeast *Saccharomyces carlsbergensis t1* has produced through fermentation 0, 15% less ethanol comparative with parental strain (Figure 5).

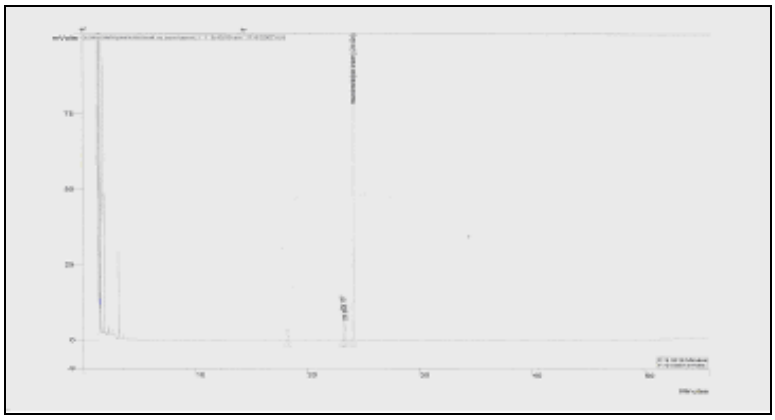

Figure 3. The chromatogram for analyze the residual glucide after the fermentation of the raffinosis by the Saccharomyces carlsbergensis strain.

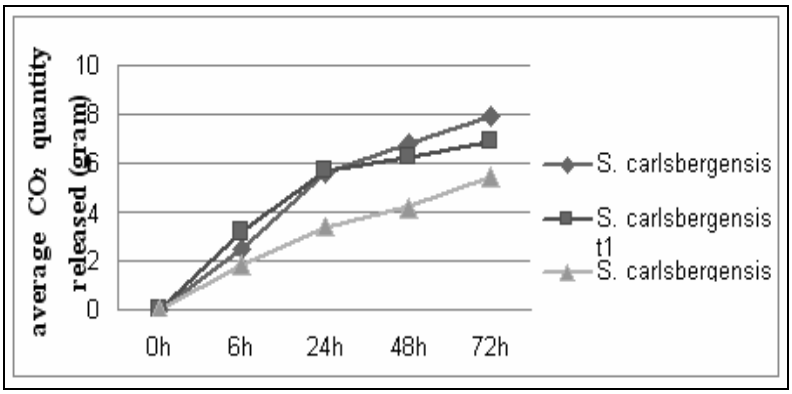

Figure 4. The average of CO_2 quantity released by fermentation.

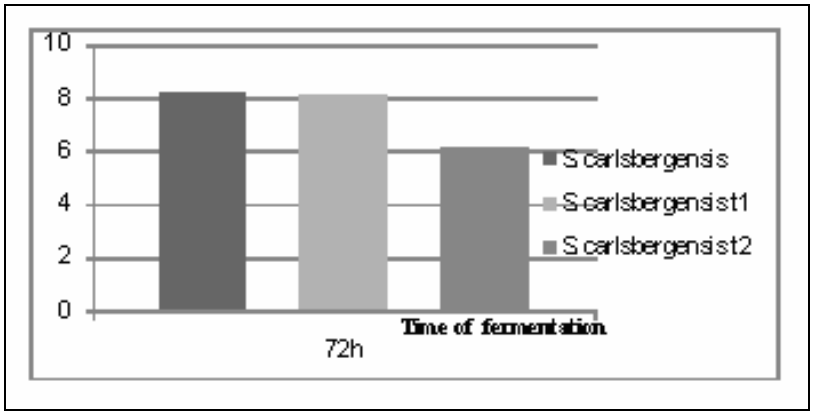

Figure 5. Concentration of ethanol produced through fermentation after 72 hours

4. Conclusions

Utilization of increase concentration of nitrogenous mixture causes a decrease of cellular Saccharomyces carlsbergensis viability and a simple change of diameter and cellular profile.

The disaccharides sucrose and melibiose and trisacharide raffinose are hydrolyzed outside the cell membrane into monosaccharide, which are then taken up by the cell.

Since beet molasses contains 40-50% sucrose and up to 2% raffinose, extracellular sugar hydrolysis is important in industrial baker's yeast production, where beer molasses often is utilized as the main carbon source. Maltose metabolism is also important for brewing.

Malt brewer's wort contains 50-60% maltose, while plain dough mainly is composed of starch, which is made available to the yeast in the form of maltose, after hydrolysis by amylases.

In contrast to the above-mentioned extracellular sugar hydrolysis, maltose is first taken up via maltose permease and then hydrolysed intracellularly by maltase into two units of glucose.Maltose permease is encoded by MALT. MAL genes cannot be induced if maltose cannot be transported into the cell (Christopher, 1998)

That can explain why the strain, *Saccharomyces carlsbergensis t2*, obtained by mutagenesis with nitrous acid, cannot fermented maltose.

In this way, it demonstrates that nitrous acid influences both rate of fermentation process and ethanol quantity produced.

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