YEAST PLASMA MEMBRANE – BIOMARKER OF YEAST QUALITY

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Rezumat

Membrana citoplasmatică a drojdiei poate fi un biomarker al calității drojdiei coreiat cu puterea de acidificare a acesteia. Pentru a determina viabilitatea şi vitalitatea drojdiei, termenii care ăi definesc calitatea, a fost folosită o versiune modificată a testului puterii de acidificare. Testul puterii de acidificare constă în determinarea a 2 parametrii: puterea de acidificare a apei (water acidification power - WAP) şi efluxul protonic indus de glucoză (glucose induced proton efflux - GIPE). S-au determinat valorile WAP şi GIPE la populații de drojdie din generația IV şi IX şi la o populație de drojdie proaspăt propagată, în condiții de depozitare la temperaturi diferite şi în prezența etanolului.

Résumé

La membrane cytoplasmique de la levure peut être un biomarquer de la qualité de la levure, corrélé au pouvoir d'acidification de celle-ci. Pour déterminer la viabilité et la vitalité de la levure- les termes qui en définissent la qualité, on a utilisé une version modifiée du test concernant le pouvoir d'acidification. Le test concernant le pouvoir d'acidification consiste dans la détermination des deux paramètres:le pouvoir d'acidification de l'eau (WAP) et l'eflux protonique induit de glucose (GIPE). On a déterminé le WAP et le GIPE aux populations IV et IX et, à une population de levures fraîchement propagée, dans des conditions de dépôt aux temperatures différentes et dans la présence de l'éthanol.

Abriss

Das Membranenzellplasma der Hefe kann eine Biomarker der Hefenqualität korreliert mit ihrer Säuerungskraft sein. Um die Lebensfähigkeit und Vitalität der Hefe zu bestimmen – Ausdrücke die die Qualität bestimmen – es wurde eine veränderte Version dem Säuerungskrafttest. Der Säuerungskrafttest besteht in der der Bestimmung von zwei Parametern: die Säuerungskraft des Wassers (WAP) und der Glukose-induzierten Protonenausfluss (GIPE). Man hat die WAP- und GIPE-Werte für Hefenstämme der III-ten und IX-ten Generation sowie auch für einen frisch verbreiteten Hefestamm bestimmt unter Stressbedingungen: Lagerung und die Gegenwart des Äthanols.

The general structure of plasma membranes is very similar in eukaryotes and in eubacteria. Differences only occur in archaebacteria which live in extreme environments at high temperatures, high salt concentrations and/or low pH. The first plasma membranes characterized in *the* early seventies were of eubacteria and eukaryotes, and it turned out that their basic composition was very similar. Therefore, the plasma membrane was also described as "unique membrane". Alternatively, plasmalemma and cytoplasmic membrane were also used.

The diameter of plasma membranes varies between 6 and 10 nm. The plasma membrane is a highly selective barrier that enables (he cell either to concentrate substances or to specifically secrete substances. Chemically the plasma membrane is a phospholipid bilayer. Phospholipid molecules are chemically unique. The basic molecule is the hydrophil glycerol (hat is covalently linked with phosphoric acid and two hydrophobic fatty acid residues by ester bridges. Therefore, phospholipid molecules are hydrophilic on one side and have a hydrophobic tail on the other. Based on this unique structure they spontaneously form membrane vesicles in aqueous solution. In contrast to membranes of eubacteria, the eukaryotic plasma membranes additionally contain sterols. About 5 to 25% of the total lipid content contributes to sterols in eukaryotic membranes. In contrast to the more flexible fatty acids, sterols are rigid planar structures. Therefore, eukaryotic membranes are more stable and much less permeable than membranes of eubacteria. Depending on the conditions

under which they have been cultivated, yeast species contain between 8-80% (w/w) lipids. Of these lipids 8-10% (w/w) can be accounted for by phospholipids and sterols (for review see Hunter and Rose 1971; Ratledge and Evans, 1989).

The membrane lipids of *Saccharomyces cerevisiae* are similar to those of other eukaryotic organisms. Yeast synthesizes sterols, and its membranes contain a typically eukaryotic mixture of phospholipids, including phosphatidylcholine and inositol-containing phospholipids. Unlike other eukaryotes, however, *S. cerevisiae* does not synthesize other lipids, and its fatty acids are predominantly 16 and 18 carbons in length (for review see Henschke and Rose, 1991; Paltauf et al., 1992).

Proteins in plasma membranes

The plasma membrane is not a rigid barrier. It is highly dynamic, that means fluid. Therefore, the term "fluid membrane" was also used. A large number of proteins are embedded in the plasma membrane, and they move by diffusion (Singer and Nicolson, 1972). Although proteins diffuse in the plasma membrane laterally, their orientation is strictly fixed, which makes membranes structurally and functionally asymmetric. The outer and inner surfaces of all known biological membranes have different components and different enzymatic activities. The unique orientation of proteins in plasma membranes is obtained as they are synthesized j and inserted into the membrane' in an asymmetric manner. This absolute asymmetry is preserved because membrane proteins do not rotate from one side of the membrane to the other and because membranes are always synthesized by growth of lire-existing membranes.

Knowing (he physiological functions of the plasma membrane in yeast, proteins in this membrane have a predictable variety of functions, such as solute transport, creating the proton-motive force across the membrane, enzymatic reactions to synthesize the cell wall, and signal transduction, mainly, by membrane-bound G-proteins. Several such proteins have been identified in yeast in recent years, but only a few of them have been studied biochemical in detail. Most intensively studied is the plasma membrane H^+-ATPase which generates the proton-motive force across the plasma membrane by extrusion of protons from the inside to the outside. Additionally, there is also increasing knowledge on sugar and *amino-acid transport*.

Transport mechanisms and distinguishing characteristics

As with most other eukaryotes, seven transport possibilities can be distinguished in *S.cerevisiae* (reviewed by R. Serrano, 1992):

- The plasma membrane H^+-ATPase, which converts the chemical energy of ATP into the physical energy of a proton-gradient;
- Ion channels for cations;
- Ion channels for anions.

Additionally, permeases exist which transport substrate molecules by:

- passive diffusion;
- proton symport;
- proton antiport;
- small molecules, such as H_2O, O_2, CO_2, ethanol and hydrophobic molecules such as undissociated carboxylic acids, can cross the membranes by diffusion, without the participation of proteins.

There is an important. difference between channels and permeases. Channels contain a fixed binding site that determines substrate specificity (Latorre and Miller, 1983). The fixed nature of their binding sites differentiates channels from permeases. The latter contain a binding site that alternates between two conformations in which it is exposed to one or the other side of the membrane. Therefore, the permeases, but not the channels, exhibit the

produced by the yeast to the medium in which it is suspended. This happens due to the addition of a specific carbohydrate, being this change directly related to the yeast vitality.

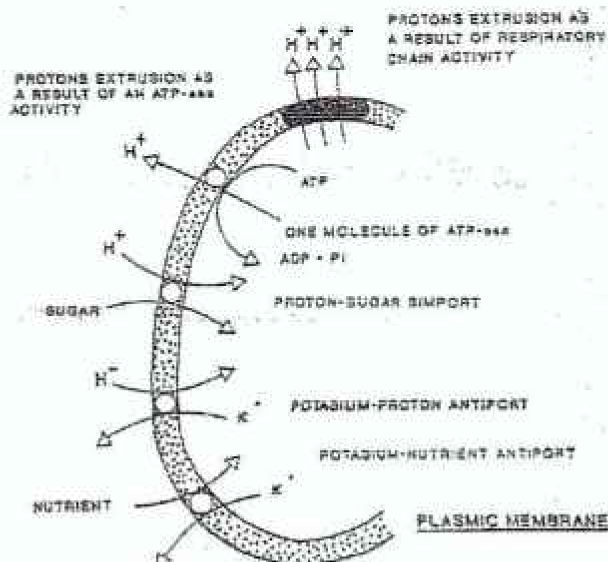


Fig. 1. Solutes' transport across plasmic membrane.

The yeast cell membrane is affected by the stresses that occur during the brewing process and particularly during storage. Starvation, ethanol (11), oxygenation (8), temperature (5), agitation, duration, and serial repitching (7) have all been identified as factors that impair fermentation performance. These stresses cause damage to the cell membrane, which, in turn, may result in reduced yeast growth, loss of viability, and a decrease in metabolic activity (10) because of membrane fluidization, lipid peroxidation (3), or loss of transmembrane potential (15). The yeast plasma membrane, therefore, is a potential biomarker for yeast quality. Many fluorescent dyes are excluded from the cell by healthy membranes. However, these dyes can freely enter dead cells with permeable membranes and bind to proteins, membranes, or DNA (6). The use of membrane-excluded fluorescent dyes, such as the hemi magnesium salt 1-anilino-8-naphthalene-sulfonic acid (MgANS) or berberine, as an alternative to the brewing industry standard, methylene blue, in determining viability has already been successfully demonstrated in previous studies (15). Similarly, the use of the anionic dye $DiBAC_4$ (2), otherwise known as oxonol, has also been demonstrated to accurately determine viability (15). Because of its negative charge, this dye cannot enter live cells, which have an intracellular negative charge because of the presence of a transmembrane potential. However, dead cells, which have leaky membranes and cannot build a membrane potential, appeared stained. The same mechanism applies for methods involving the measurement of capacitance for determination of the live cell concentration (4). Furthermore, it has been demonstrated that a measure of capacitance in combination with the levels of fluorescence emitted by dead cells stained with oxonol resulted in an accurate determination of viability (2).

Yeast plasma membrane has not only been proved useful in determining viability but also vitality. Indeed, plasma membrane proton efflux can be measured by using the acidification power test, and this has been demonstrated to correlate to fermentation performance (9). The test was first designed by Opekarova and Sigler in 1982, and

numerous improvements have been made since then. Recently, controls have been added to render the test more accurate and reproducible, resulting in the measurement of two parameters: glucose acidification power (GAP) and water acidification power (WAP) (12). The WAP is the change in pH when cells are resuspended in water for 20 min and corresponds to the concentration of intracellular glycogen (14). The GAP represents the change in pH when cells are resuspended in water for 10 min followed by 10 min in glucose. During this last 10 min, both glucose-induced and passive proton effluxes are measured. To obtain the net glucose-induced proton efflux (GIPE), the WAP is deducted from the GAP. Therefore, the GIPE represents the ability of the cells to use extracellular reserves, without taking into account the utilization of intracellular reserves (12).

The "acidification power" of the cell is provided by the proton pump enzyme H^+ATPase, encoded by the *PMA1* gene. This enzyme is located in the plasma membrane of yeast and regulates intracellular pH by pumping protons out of the cells. This efflux of protons creates an electrochemical gradient that drives the transport of nutrients into the cell; in this way, *H^+ATPase activity is essential for brewing yeast to utilize wort. Levels of* adenosinetriphosphatase (ATPase) activity change with various stresses (16), with consequent changes in the extent of proton efflux. Thus, the capacity of a brewing yeast cell to utilize wort is directly linked to its plasma membrane integrity and H^+ATPase activity.

The GIPE represents the differentiation between two components known as WAP and GAP.

The WAP reflects the extent of the passive proton efflux in the absence of exogenous carbon source and, therefore, *reflects the utilization of the cell's reserve carbohydrates for* metabolic activity maintenance. It has been postulated that passive proton efflux correlates with intracellular levels of glycogen (9).

The GIPE is dependent on the activity of the ATPase proton pump located in the plasma membrane, which promotes the efflux of protons as a result of glycolysis. Protons are extruded to enable the cell to counteract increases in intracellular pH, thereby maintaining the pH buffering capacity of the cytoplasm. Damage to the membrane or the ATPase enzyme will, therefore, cause changes in proton efflux. In support of this hypothesis, the GIPE was observed to be inversely correlated with intracellular levels of trehalose (14) and since accumulation of this reserve carbohydrate occurs in the presence of yeast-handling stresses due to the activation of the stress response element (STRE)-induced global stress response (13), the GIPE represents an indirect determinant of the stressed phenotype in brewing strains.

WAP as an Indicator of Yeast Quality

Levels of glycogen are critical at the beginning of the fermentation. Yeast cells use glycogen in the presence of oxygen to build up the pool of sterols necessary for the cells to divide and start fermenting. Glycogen levels can be affected by handling and are indeed significantly reduced during starvation and in the presence of various stresses (7) and oxygenation (1).

Starvation occurs when a carbon source is not available exogenously and cells utilize their intracellular reserves to maintain metabolic activity. Furthermore, starvation is likely to occur during slurry storage, and levels of glycogen have been observed to drop, sometimes drastically, during residence in a storage tank (brink). To determine the impact of long-term starvation on passive proton efflux, the WAP was measured for ale yeast cell populations (freshly propagated yeast, generation IV yeast, generation IX yeast) re-suspended in water under agitated conditions at 5°C and 1°C.The WAP decreased progressively after each day of starvation (table 1, figure 2). The results indicate that the levels of intracellular reserves are depleted more rapidly during starvation at 5°C.

Lower levels of glycogen in pitching yeast extend the lag phase in the fermenter and affect overall fermentation performance, suggesting that this intracellular carbohydrate is a good indicator of stored yeast quality (1). However, direct measurement of glycogen levels requires complex enzyme assays involving lengthy methods and sometimes, specialized equipment. Since the WAP indirectly measures glycogen levels and is very operator friendly and rapid, it is postulated that this assay may be utilized to determine the potential of yeast slurry to perform.

Table 1: WAP values during storage period at 5°C

Yeast / storage period	0	1 day	2 days	3 days	4 days	5 days	6 days
WAP values at IX generation yeast	0.28	0.2	0.22	0.22	0.08	0.02	0
WAP values at IV generation yeast	0.48	0.47	0.4	0.4	0.32	0.2	0.15
WAP values at freshly propagated yeast	0.37	0.35	0.32	0.32	0.21	0.1	0.1

Figure 2: WAP values during storage period at 5 °C

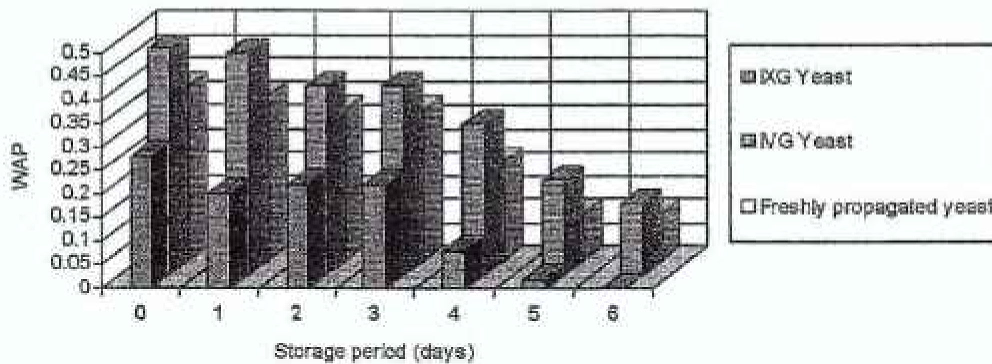
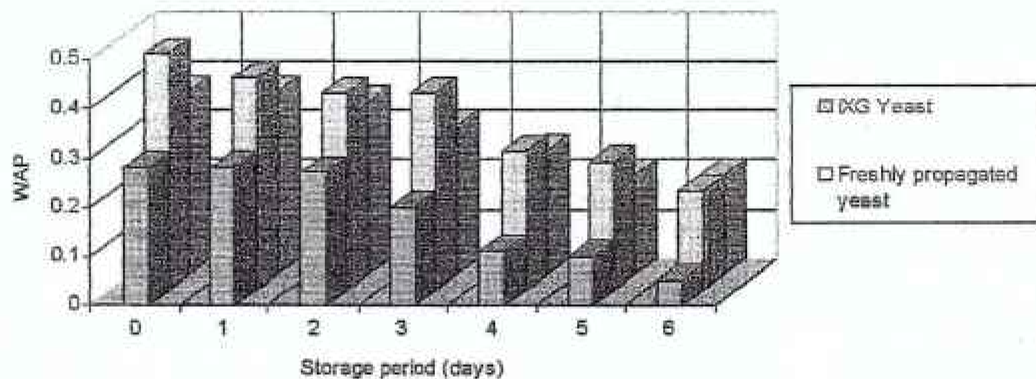


Table 2: WAP values during storage period at 1°C

Yeast / storage period	0	1 day	2 days	3 days	4 days	5 days	6 days
WAP values at IX generation yeast	0.28	0.2	0.22	0.22	0.08	0.02	0
WAP values at IV generation yeast	0.48	0.47	0.4	0.4	0.32	0.2	0.15
WAP values at freshly propagated yeast	0.37	0.35	0.32	0.32	0.21	0.1	0.1

Figure 3: Wap values during storage at 1 °C



More affected are IX generation yeast and freshly propagated yeast. The WAP value at IV generation yeast indicates an increased level of glycogen comparative to freshly propagated yeast. These data support previous observations, where glycogen increased depending on generation number.

Pitching yeast should be stored at 1°C rather than at 5°C for preventing the lose of its vitality.

GIPE as an Indicator of Yeast Quality

Stress may occur during yeast handling, and serial repitching has been considered as inducing a "repeated stress injury"' in production slurries (7). In particular, ethanol represents a chemical stress, especially during high-gravity brewing when concentrations can range from 7 to 14% (v/v). It has been postulated that ethanol causes an increase in proton influx because of enhanced membrane permeability and the accumulation of unsaturated fatty acids. The increase in the ratio of unsaturated to saturated fatty acids results in the fluidization of the plasma membrane, causing a "leakiness" that has been previously linked to ion and enzyme release. Furthermore, repression of expression of the *PMA1* gene that encodes the plasma membrane H^+ATPase has been reported, which may account for the inhibition of the activity of the ATPase proton pump that is normally associated with ethanol toxicity.

The *impact of 4, 8, and 12% ethanol on the GIPE of yeast populations (freshly* propagated yeast, generation IV yeast, generation IX yeast) was determined at 2, 4, 6 h and initial.

The GIPE decreased significantly after 2 h of exposure to all ethanol concentrations and continued to decline on further exposure up to 6 h (figure 4, 5, 6, table 3), confirming previous observations that sublethal concentrations of ethanol influence plasma membrane functionality.

The GIPE values of freshly propagated yeast (which initially showed lower GIPE values than the IX generation yeast) after an exposure time of 2 hours at ethanol stress, are higher than those of the IV generation yeast at the same exposure time.

This indicates a higher adaptation capacity at ethanol stress than of the freshly propagated yeast. However the yeast loses from its reaction capacity at ethanol stress due to the partial loss of membrane functionality.

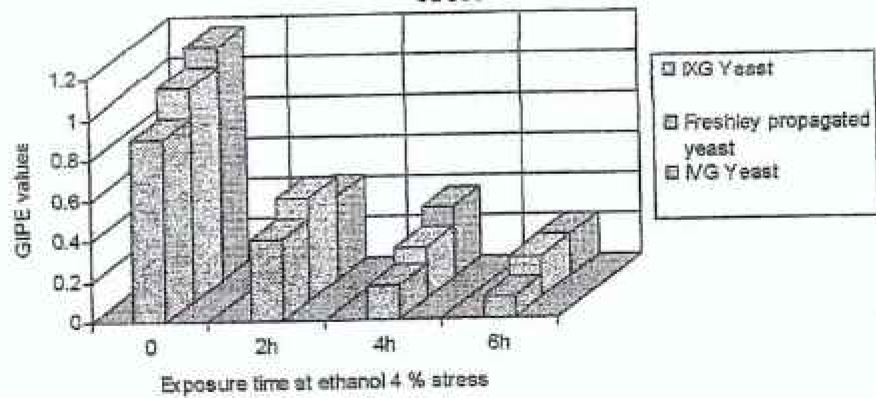


Figure 4: GIPE values at yeast populations exposed to ethanol 4% stress

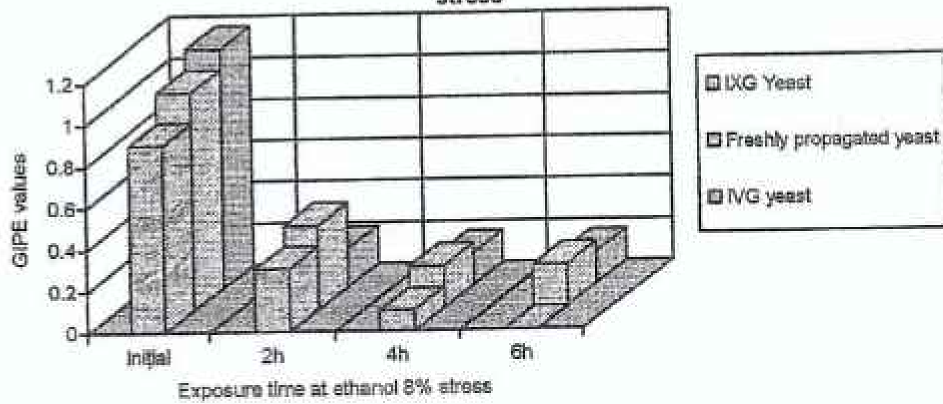


Figure 5: Gipe values at yeast populations exposed to ethanol 8% stress

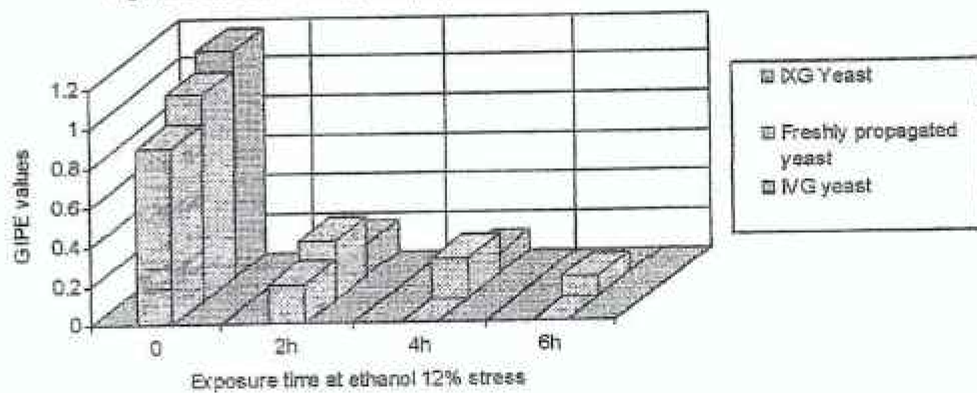


Figure 6: GIPE values at yeast populations exposed to ethanol 12% stress

Table 3: Gipe values for yeast populations exposed at ethanol stress

Exposure time at ethanol stress	Ethanol concentration, %	GIPE values		
		Freshly propagated yeast	IV generation yeast	IX generation yeast
0	-	1,05	1,15	0,9
2h	4	0,5	0,41	0,4
	8	0,4	0,18	0,3
	12	0,3	0,16	0,2
4h	4	0,25	0,34	0,17
	8	0,2	0,15	0,1
	12	0,2	0,1	0
6h	4	0,2	0,2	0,1
	8	0,2	0,15	0
	12	0,1	0	0

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