Acidification Power (AP) Test and Similar Methods for Assessment and Prediction of Fermentation Activity of Industrial Microorganisms

Test acidifikační síly (AP) a příbuzné metody pro stanovení a předpověď fermentační aktivity průmyslových mikroorganismů

Karel SIGLER
Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4 / Mikrobiologický ústav, Akademie věd České republiky, Vídeňská 1083, 142 20 Praha 4
e-mail: sigler@biomed.cas.cz

Reviewed paper / Recenzovaný článek

Keywords: proton extrusion, acidification power test, cell vitality, industrial microorganisms
Klíčová slova: vypuzování protonů, test acidifikační síly, vitalita buněk, průmyslové mikroorganismy

1 INTRODUCTION

Many microorganisms (yeasts, lactic acid bacteria) acidify outer medium during fermentative processes that take place, e.g., during dough leavening, cheese or wine making and during beer brewing. The surge of external acidification taking place on resuspending cells in a new medium or adding a metabolic substrate belong to the so-called “early events” that occur before any measurable change of the substrate, constitute an immediate response of the culture exposed to a new environment (Quain, 1988) and have been shown to be very useful as significant indicators of the physiological status of the culture and key events necessary for normal fermentation (Riis et al., 1995). Transport studies performed in the 1980’s (Sigler et al., 1981a,b; Sigler et al., 1983; Pascual et al., 1983) showed that the ability of yeast cells to excrete acidity from several sources (proton extrusion by membrane H+–ATPase, H+∕K+ exchange, production of CO2 and organic acids) is a strictly controlled process that reflects very closely the metabolic vigor of the cells or, in other words, their vitality under given conditions. The results of these studies led to the elaboration by Řepkarová and Sigler (1982) of the acidification power (AP) test (see also Sigler and Höfer, 1991a,b; 1997), which provided a fast and methodologically simple means of assessing how various factors (previous history of cell culture, stress factors such as starvation, temperature, osmotic pressure, freeze drying, chemical stress imposed by components of fermentation media, preservatives and others) affect the ability of cells to utilize a sugar substrate such as glucose and, consequently, perform successful and technologically profitable fermentation. Still more importantly, the test made it possible to predict the outcome of the fermentation process conducted with a certain batch of cells, and thus prevent sluggish or stuck fermentations.

Because it offered an easy-to-perform assay for assessing and, above all, predicting the metabolic vigor of wet starter cultures to be used for industrial purposes, the acidification power test became subsequently widely used in the brewing (Femandez et al., 1991, 1993; Isertentant et al., 1996; Kara et al., 1988; Lawrence, 2002; Peddie et al., 1991; Šusta et al., 1984; Van Zandyczke et al., 2001) and baking industries (Collar et al., 2005; Higgins et al., 2001; Vincent et al., 1999), in wine-making (Maffeio-Ferreira et al., 1990) and in other biotechnology branches, e.g. for determining the freshness of rice (Shindo et al., 2000). It was also employed for evaluating the metabolic prowess of immobilized (Shen et al., 2003; Wackerbauer et al., 2002) and freeze-dried yeast (Peddie et al., 1991), of newly generated baker’s yeast strains (Higgins et al., 2001) and non-saccharomyces yeasts such as Zygosaccharomycyes rouxii (Yoshikawa et al., 1995).

The test has had several advantages compared with previously used fermentative power assays (e.g. Pearce, 1969); it is fast (the result can be achieved within 60–80 min; Gatto et al., 1993) and requires only a basic equipment (a centrifuge and a temperature-compensated pH meter) available in most food industry laboratories. Because of its simplicity and satisfactory reproducibility, it has been used as a rejection criterion for assessing the technological quality of starter cultures and its use extended from brew- ery to cider production, bakery products manufacture and cheese making based on lactic acid bacteria (Gatto et al., 1993; International Dairy Federation Guideline, 1995; Rechinger and Siegumfeld, 2002; Riis et al., 1995; Semjonovs et al., 2004) and other microorganisms.
2 PROCESSES INVOLVED IN ACIDITY EXTRUSION

The various processes involved in both spontaneous and substrate-induced acidification of yeast suspensions have been analyzed in detail by Sigler and Höfer (1991a). In yeast, extracellular acidification occurs under both aerobic and anaerobic conditions and is fuelled by a variety of glycolytic as well as respiratory substrates, both endogenous and exogenous. Respiratory substrates under conventional conditions (in a medium containing dissolved oxygen at normal partial pressure) cause very low acidification due to the oxygen limitation that sets in very quickly under these conditions. Under intensive aeration the acidification caused by ethanol is fully comparable with that caused by glucose. Even without any exogenous substrate, addition of $H_2O_2$ concentrations $>10$ mmol/L to aerated derepressed yeast suspension causes a comparable acidification that persists until necessary endogenous substrates are consumed in respiratory processes. This acidification is suspended by $H^+\text{-ATPase}$ inhibitors and is associated with concomitant $K^+\text{-influx}$ (Sigler and Höfer, 1991a,b).

The participating processes include $CO_2$ production from fermentative or respiratory processes, extrusion of protons by the plasma membrane via the $H^+\text{-ATPase}$, efflux of acidic metabolites (such as succinate or lactate, and $K^+/H^+$ exchange. The contributions of individual processes to the overall acidification course and intensity appear to depend strongly on measurement conditions (substrate and cell concentration, aeration, etc.). Thus Myers et al. (2005), on the basis of the lack of effect of $H^+\text{-ATPase}$ inhibitors such as diethylstilbestrol and vanadate, reported that glucose-supported acidification in aerated yeast suspension is almost entirely due to $CO_2$ production by the cells, the $H^+\text{-ATPase}$ playing no role in the process. Sigler et al. (1991b), on the other hand, found under their experimental conditions a strong inhibition of acidification when $H^+\text{-ATPase}$ was inhibited by diethylstilbestrol and asserted that the enzyme plays an important part in the acidification process.

Machnicka et al. (2004) described a set of $ACI$ genes apparently responsible for signaling amino acid deprivation in yeast cells in which part of the Krebs cycle intermediates have to be used for amino acid synthesis via the glyoxylate cycle. Mutants in these genes excrete protons instantaneously on being suspended in unbuffered water in the absence of any external carbon source, excreting citrate, aconitate, succinate, fumarate or malate and reaching external pH levels below that produced on glucose addition.

3 AP TEST

The test is very simple. In the initial part, pH is measured 10 min after suspending the cells in water without exogenous sugar. This spontaneous acidification power, given by the $\Delta pH_{10}$ value, reflects the metabolism of endogenous substrates and $CO_2$ production. In the second part, the cell suspension is supplied with glucose, which causes an additional strong acidification of the external medium that is gauged by measuring the pH value of the suspension after another 10 min – $\Delta pH_{20}$. The magnitude of this glucose-induced $H^+$ efflux reflects the fermentative ability of the yeast while the sum of $\Delta pH_{10}$ and $\Delta pH_{20}$, termed the acidification power (AP) reflects the overall vitality of the cells (Opekárová and Sigler, 1982). The intensity of the substrate-induced acidification is controlled by external pH. If this control is abolished by keeping the pH artificially constant, e.g. in a pH-stat, the net proton efflux increases up to 100-fold. According to Castrillo et al. (1995), this is then independent of the buffering capacity of culture medium. In a chemostat culture, it is closely associated with the nitrogen uptake rate.

Sigler et al. (1981a) showed in laboratory yeast strains that the extent of acidification observed in aqueous yeast suspension after glucose addition strongly depends on cell concentration, increasing with rising suspension density and reaching a constant level at a cell concentration of about $50-60$ g/l. Walker and Kolyer (1985) noted that the specific rates of many processes in diverse microorganisms, i.e. the rates referred to a single cell, drop markedly with increasing suspension density, leveling off at a certain cell concentration characteristic for the given microorganism and the type of metabolic or transport process. The signal substance suppressing the activity of individual cells at higher cell concentrations was suggested to be $CO_2$, which has been known to have multifarious affects on cells (Das et al., 1990; Jones and Greenfield, 1982; Mitz, 1979; Oura, 1977).

Hence, the AP test has to be performed at these saturation cell concentrations because otherwise small differences in cell concentration in individual samples (unavoidable if the cells are used as slurries or wet pellets) can lead to large differences in AP. Except for special cases (the vitalitration of Rodrigues et al., 2003), the cell concentration used in most studies to ensure saturation conditions was, in terms of the industrially most suitable use, equal to a minimum of 50 $g$ packed yeast pellet per l.

The dependence of the AP value on the concentration of glucose has likewise a saturation character, and the sugar concentration used should be high enough to ensure essentially constant $H^+$ efflux irrespective of minor variations in sugar level. In this respect, different studies were performed under widely varying conditions, the glucose concentrations ranging from $1.7$ to $9$ $\mu$mol/L, the latter value being proposed by Opekárová and Sigler (1981) as truly saturating.

Gabriel et al. (2008) optimized the AP by storing the yeast slurry at $2^\circ$C under beer. Under these conditions the AP remains constant for up to 6 days. Sample equilibration to room temperature and washing in deionised water ensured that the final yeast pellet kept its AP for up to 6 h at room temperature under water and the AP test did not need to be performed immediately after yeast collection. The maximum acidification produced by given yeast was determined in a sample containing $\geq 5\%$ glucose and $\geq 1.5$ $\mu$mol/L yeast wet weight. Cell flocculation and/or sedimentation that can distort AP results can be prevented by stirring the sample at $200$ rpm. The lowest AP of yeast cropped from cylindroconical tanks was displayed by the first cropped fraction. Variations in pitching yeast vitality and their effect on the outcome of a brewery fermentation can be masked by variations in pitching rate, wort composition, ambient conditions in the cylindroconical tanks and other factors.

4 ACIDIFICATION POWER AND FERMENTATION VIGOR

4.1 Brewing industry

The $\Delta pH_{10}$ value has been suggested to be related to the growth potential of a given pitching yeast lot (Kara et al., 1988). When the AP test is performed at intervals during the fermentation process, the evolution of this initial endogenous $H^+$ extrusion was found to be identical to the evolution of glycogen level in the cells (Imai, 1999). Although it was found by some authors to be less reproducible than the subsequently measured glucose-induced and mostly $H^+\text{-ATPase}$-mediated proton efflux, this stage is essential to equilibrate the pH of the cells (Van Zandycke et al., 2001). On the other hand, the glucose-induced acidification power $\Delta pH_{20}$ is an indicator of the glycolytic flux rate (Mathieu et al., 1991; Imai et al., 1994). In an overall AP value of brewery yeast of, say, 2.5 the $\Delta pH_{10}$ is typically around 0.5, the $\Delta pH_{20}$ being around 2 (see, e.g., Kara et al., 1988).

4.2 Baking industry

In baking industry, acidification power was shown to be inversely related to the specific volume of breads and the degree of dough cohesiveness in fresh pan breads doped with microbial transglutaminase (Collar et al., 2005).

Two types of baker’s yeast are used in the modern baking industry: strains with efficient maltose metabolism optimized for use in unsugared dough and osmotolerant strains specialized for use in sweet doughs with sugar concentrations of up to $30\%$ ($w/w$) of flour. A cycling between growth on galactose and growth on maltose was found to enrich the yeast culture in efficient maltose utilizers. The maltose utilization phenotype was determined by the AP test (Higgins et al., 2001).

4.3 Milk processing – lactic acid bacteria

Lactic acid bacteria (LAB) are widely used as starter cultures for fermentation of milk, vegetables, and meat. Gatto et al. (1993) modified the AP test for use with the lactic acid bacteria and tested its applicability on dried and rehydrated Streptococcus cremoris/S. lactis and Lactobacillus acidophilus. The assay was found to be considerably faster and methodologically sim-
pler than, e.g., the classical Pearce test based on small scale fermentation in milk (Pearce, 1969). The non-continuous substrate acidification with lactic acid bacteria is negligible; the requisite 10-min period was retained, then lactose was added and the AP value was calculated as a difference between the starting pH of 6.3 and the ΔpH measured 10 min after sugar addition. The AP values of the bacteria were markedly affected by cell washing and rehydration at different temperatures, by the temperature at which the AP test was performed, and was much higher with lactose than with sucrose as acidification substrate. A positive correlation was observed between the AP value and the results of the Pearce test. With frozen, frozen and stressed as well as lyophilized *Lactobacillus delbrueckii* ssp. *bulgaricus* cultures the AP test was found to be more sensitive than the standard acidification test or flow cytometry (Riis et al., 1995).

Rechinger and Siegmundfeld (2002) prepared *Lactobacillus delbrueckii* ssp. *bulgaricus* cultures with different viable cell counts by mixing frozen and lyophilized cells in different ratios. The AP values correlated well with the degree of vitality. This linear correlation between AP values and the Pearce test data was not confirmed by Riis et al. (1995) who, however, found a considerable impact of temperature stress on the AP values. According to the authors, the cell material was not freeze-dried and therefore the freeze storage media cultures do not seem to influence the ability of the bacteria to spontaneously utilize their endogenous carbohydrates while it reduces the ability of the bacteria to utilize exogenous sugars. Both flow cytometry and classical fermentation of reconstituted skim milk failed to reveal these effects.

## 5 OTHER TESTS BASED ON PROTON OR ION RELATIONSHIPS IN CELLS

A number of studies were devoted to assessing the merits and shortcoming of the AP test. Thus Willetts et al. (1997) and Seward et al. (1996), when testing the vitality of cider yeast grown in the presence of different alcohols, showed the AP value to be a sensitive indicator of vitality superior to, e.g., the adenylate charge values. In studies on immobilized brewery yeast, the AP was a useful measure of cell vitality but was reported to suffer in some cases from low reproducibility (Wackerbauer et al., 2002). Since the AP test should serve primarily as a rejection criterion for fermentation-incompetent cells, it should be employed with caution for, e.g., distinguishing between several highly fermentation-competent strains having high AP values (Iserentant et al., 1996). In this situation the predictive power, i.e. the key to how the cells will behave when exposed to future stress (e.g. high ethanol, environment, etc.) is lost because at a momentary good condition – replete with endogenous substrates, with high adenylate charge, etc. – will, unless exposed to a stress of sufficient intensity and duration, not provide any cue as to their possible behavior when stressed. The methods stated to be suitable for these cases are, e.g., the assay of intracellular pH as a metabolic indicator using suitable fluorimetric or other techniques (Imai et al., 1994); a microtitre plate modification of the AP test was used for a speedy estimate of the metabolic prowess of baker’s yeast (Vincent et al., 1999). The rising awareness of the significance of not only protons, but ionic homeostasis in general, brought yeast vitality assessment methods that use as the key parameter, e.g., the rate of magnesium release during wort fermentation (Mochaba et al., 1997, 1998). A detailed whole-genome analysis of the significance and role of various ions in yeast cells, the so-called ionome, has been published by Eide et al. (2005).

## 6 SOME FACTORS AFFECTING THE ACIDIFICATION RATE

Among the factors affecting the acidification rate are, e.g., high ethanol concentration and high wort osmolality encountered during high gravity brewing (HGB).

Osmotic pressure was shown to affect strongly the rate of acidification in *S. cerevisiae* (de Maranon et al., 2001). Hypersmotic shock caused by both permeant and impermeant solutes lowers the acidification rate; a total inhibition of the process takes place when the size of the osmotically shrunk cells reaches 50% of initial cell volume. The osmotic pressure exerted by permeant solutes (glucose, xyitol, sorbitol) is about 20 MPa while impermeant solutes (PEG 600) exert an equal effect at 8.5 MPa.

Using the AP test, yeast from high-gravity (HGB) fermentation was found to be unsuitable for re-pitching since the cell membrane is adversely affected by the high ethanol concentration (Van Zandyczke et al., 2001). The proton extrusion rate was found to be markedly inhibited during high gravity and very high gravity browning of *S. pastorianus*, the inhibition being stronger at later stages of HGB and VHG wort fermentation and being given to a large extent by high ethanol concentration (Yu et al., 2011). Likewise, the negative effects of HGB were found to be unconnected with osmotic effects of high sugar concentrations up to 25% (w/v) and have been attributed entirely to ethanol concentration (Hammond et al., 2001). They were reported to be largely mitigated by the yeast peptide complex (YPC) obtained by alcoholic extraction of yeast (Debourg, 2002).

Sigler et al. (2009) mimicked HGB conditions by using standard wort supplemented with sorbitol to determine the net effect of wort osmolality without the influence of ethanol. They described the effect
of osmolarity alone on fermentation course, wort attenuation, yeast flocculation and acidification power.

The effect of acid washing on the acidification was studied by Fernandez et al. (1995).

Duarte, O. Production of ciders from apple juice, a number of higher alcohols are produced by the cells that may pose considerable chemical stress. Although the yeast strains used in cider fermentations are as a rule ethanol-tolerant, high ethanol concentrations also can have deleterious effects on cells. The presence of 10% v/v ethanol, 0.05% hexan-1-ol and 0.25% 2-phenylethanol during aerobic growth and fermentation of cider yeast reduced both the growth rate and the AP value (Seward et al., 1996). The combinations of ethanol with either of the two other alcohols were found to act synergistically to cause a pronounced decrease in viability and acidification power. These combinations are assumed to produce membrane lesions that adversely affect cider fermentation. Yeast repitching in cider fermentation (Dinsdale et al., 1995, 1999) brings about a considerable loss of vitality but a little loss of viability.

Acknowledgements

The work was supported by Institutional Research Concept V061388971.
Acidification power (AP) test and similar methods for assessment

Do redakce došlo / Manuscript received: 17.04.2013
Přijato k publikování / Accepted for publication: 09.06.2013

25. PIVOVARSKO-SLADAŘSKÉ DNY

termín: 7. listopad 2013
místo: Pivovar Staropramen, Praha
organizátor: VÚPS, a.s., VŠCHT v Praze, Pivovary Staropramen a.s.
aktuální informace na www.pivovarskedny.cz

K akci vyjde dvojčíslo 10–11, kde budou fulltexty přednášek ve formě recenzovaných publikací
Podrobnější informace přineseme v příštích číslech

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ