# Physiological State of Reused Brewing Yeast

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**Abstract**

Kordialik-Bogacka E., Diowksz A. (2013): **Physiological state of reused brewing yeast**. Czech J. Food Sci., **31**: 264–269.

In brewing, yeast may be reused many times. A number of yeast repitchings differs significantly among the breweries. Adjusting the number of times a strain may be serially repitched is of great importance for quality and consistency of final products. The fermentative and physiological characteristics of the yeast culture used in successive laboratory scale fermentations were determined. Yeast physiological state was assessed by the measurement of the levels of intracellular carbohydrates. In our investigation there were not any detectable changes in yeast capacity to ferment. No significant variation in the production of flavour compounds was found either. However, intracellular glycogen and trehalose contents were dependant on the yeast strain, generation number and wort gravity. Nevertheless, an alteration in the yeast physiological condition during serial repitchings occurred in a different mode than in previous studies confirming that the impact of serial repitchings is strain and medium dependent to a large extent.

**Keywords**: flavour compounds; glycogen; serial repitching; trehalose; yeast

On the completion of brewery fermentation yeast is harvested from the fermentation vessel and after a short period of time reinoculated into a fresh wort batch. A number of times yeast can be reused depends on a variety of factors, but mainly on the individual strain, quality of the cropped yeast, original wort gravity and company policy. There is a big variation in a number of yeast repitchings among the breweries. In some breweries a lager brewing yeast culture is used 2–3 times while in others even 7–9 times for fermentation of wort at similar original gravity (O'Connor-Cox 1997). It has also been reported that lager yeast can be reused even up to 20 times (Powell *et al.* 2003; Stewart 2009).

Fermentation performance is affected by external factors, such as wort gravity, wort oxygenation and clarity, pitching rate and temperature. The increased osmotic and hydrostatic pressure, elevated alcohol concentration and modified nutrient balance have a profound influence on yeast performance. And such conditions are often met in modern brewing. With increasing wort gravity the number of yeast generations (cycles) that can be employed is reduced.

During the course of serial repitching yeast physiological condition may be deteriorated and microbial contamination can occur. Yeast physiological state prior to pitching determines the consistency of fermentation and product quality. Physiological condition can be assessed by the determination of specific yeast cell components important for fermentation activity (Axcell & O'Connor-Cox 1996). Two major intracellular storage carbohydrates – trehalose and glycogen – are commonly used physiological marker substances.

Trehalose protects the cell against stress induced by osmotic pressure, ethanol, high and low temperature and desiccation (Odumeru *et al.* 1993; Schlee *et al.* 2006). It is an important stress indicator in brewing yeast cultures. It also plays a role during the initiation of the cell cycle, as it quickly supplies a carbohydrate and serves as an energy source.

Glycogen is the major reserve energy storage material in yeast cells. During the first 6–8 h of wort fermentation there is a rapid utilisation of in-

tracellular glycogen, which is directly proportional to the synthesis of lipids and sterols, used by the cells to produce membranes during cell division (Stewart 2009). Once nutrients are consumed and cell division terminates, glycogen accumulates (Quain & Tubb 1982; Stewart 2009). It is important that maximum levels of intracellular glycogen are present in the yeast culture when it is harvested for storage, prior to being repitched into a subsequent wort fermentation. Depleted glycogen levels lead to incomplete fermentation.

The good yeast quality is a requisite to obtain consistent beer with correct flavour balance. A wide range of esters and higher alcohols which are produced as a result of yeast metabolic activity contribute to beer flavour. They are considered positive flavour attributes of beer, although their too high concentration can impart an unpleasant aroma.

In this study the effect of serial repitching on yeast physiological condition was examined.

## MATERIAL AND METHODS

***Yeast strains***. Two bottom-fermenting yeast strains of *Saccharomyces pastorianus* designated 308 and B4 (LOCK 0100 and 0075) were used. Yeast was grown in 10°Plato wort for 48 h at 25°C on an orbital shaker at 350 rpm. The cells were harvested by centrifugation (2500 *g*, 5 min) and pitched into 500 ml cylinders containing 400 ml of hopped malt wort at original gravity of 10°Plato or 15°Plato collected from a commercial brewery. The pitching rate was $2 \times 10^7$cells/ml. This initiated the first fermentation cycle.

***Serial repitching***. Yeast strains were serially repitched by reusing a proportion of biomass removed at the end of each fermentation. Each time yeast was pitched into fresh wort to a final concentration $2 \times 10^7$cells/ml. Fermentations were carried out at 9°C for 9 days (10°P wort) or 14 days (15°P wort). All fermentations were performed at the same conditions using the same wort. For each strain ten successive fermentations were carried out with 10°P wort and eight ones with 15°P wort. Each fermentation was conducted in three replicates. Beer was then centrifuged to remove yeast (2500 *g*, 5 min, 9°C). One portion of yeast biomass was used for inoculating the next batch of fresh wort and the other, after chosen fermentations, for the determination of yeast viability with methylene blue as well as glycogen and trehalose content.

Fermentation performance of yeast slurries was also assessed in terms of beer attenuation and flavour profile of produced beer.

***Glycogen and trehalose determination***. Physiological conditions of two yeast strains after propagation and upon completion of successive fermentations were studied. It was done by determining the content of glycogen and trehalose in yeast cells. For the measurement of glycogen and trehalose content the method described by Jenkins *et al*. (2003) was applied.

***Volatile ester and higher alcohol determination***. After successive fermentations beer flavour was evaluated using Agilent Technologies 6890N gas chromatograph (Agilent Technologies, Inc., Santa Clara, USA), equipped with a flame ionisation detector (FID). Higher alcohols (propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol) and esters (ethyl acetate, ethyl butyrate, isoamyl acetate, ethyl caproate and ethyl caprylate) were determined (Institute of Brewing 1997).

***Statistical analysis***. Statistical significance of the data was determined by Student's *t*-test. A *P*-value below 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

In brewing yeast may be reused for fermentation several times. However, after a certain time deterioration of cropped yeast is observed and a newly propagated yeast culture has to be applied. Lower yeast slurry quality is directly related to poorer fermentation performance and consequently to lower quality of final beer. Alterations in fermentation performance can already be assessed by the measurement of attenuation, which provides information on the extent to which wort sugars can be used. It describes yeast ability to reach a certain attenuation limit.

Yeast fermentation characteristics were evaluated in ten successive fermentation cycles of 10°P wort and eight cycles of 15°P wort. It was observed that attenuation profiles did not differ significantly in successive fermentations. In the case of fermentation of 15°P wort the final gravity amounted to 3.5 ± 0.2°P (strain 308) and 5.1 ± 0.2°P (strain B4). When 10°P wort was fermented, the final gravity was 3.0 ± 0.1°P (strain 308) or 4.0 ± 0.2°P (strain B4). Thus the yeast ability to utilise sugars remained stable over time.

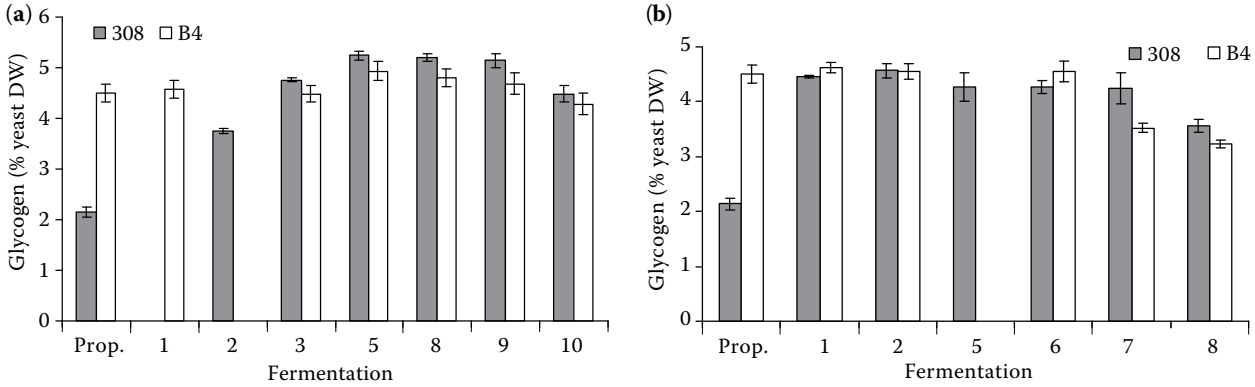


Figure 1. Glycogen content in yeast cells of strains 308 and B4 after propagation and successive fermentation of 10°P (**a**) and 15°P (**b**) wort

The viability of yeast cells after successive fermentations did not change significantly either. The amount of viable cells was always higher than 98%.

Among many parameters cited in the literature to assess the yeast physiological state, the measurement of intracellular glycogen and trehalose content was chosen in this study.

### Glycogen content in repitched yeast

To examine the relative quality of slurries for each strain, samples were collected after propagation immediately before pitching into the first fermentation.

During propagation in the presence of oxygen glycogen is utilised to synthesise sterols and lipids for the membrane production. It is unusual that this phenomenon was not observed for strain B4 (Figure 1). Glycogen content in yeast strain 308 after propagation was much lower than after fermentation processes.

During serial repitching of strain 308 used for fermentation of 10°P wort the glycogen level after initial fermentation processes was lower than after final ones (Figure 1a). There were not any differences in glycogen content among generations 5, 8, and 9. However, the statistically significant decline in glycogen content was observed for generation 10 ($P < 0.01$), which can suggest deterioration of the yeast physiological state.

There were not any statistically significant changes in glycogen content during serial repitching of strain B4 until generation 10. Glycogen content in yeast collected upon completion of fermentation 10 was significantly lower than in cropped yeast sample 5 ($P < 0.05$), when glycogen content was the highest.

In the case of fermentation of 15°P wort the lowest glycogen content was in yeast cropped from the last performed fermentation 8 for both strains (Figure 1b). For strain B4 there was a statistically significant change in glycogen level starting from yeast sample 7 ($P < 0.001$). Earlier there were not any statistically significant differences in glycogen content after fermentations 1, 2 and 6. In analysed samples for strain 308 the glycogen level was similar until generation 8.

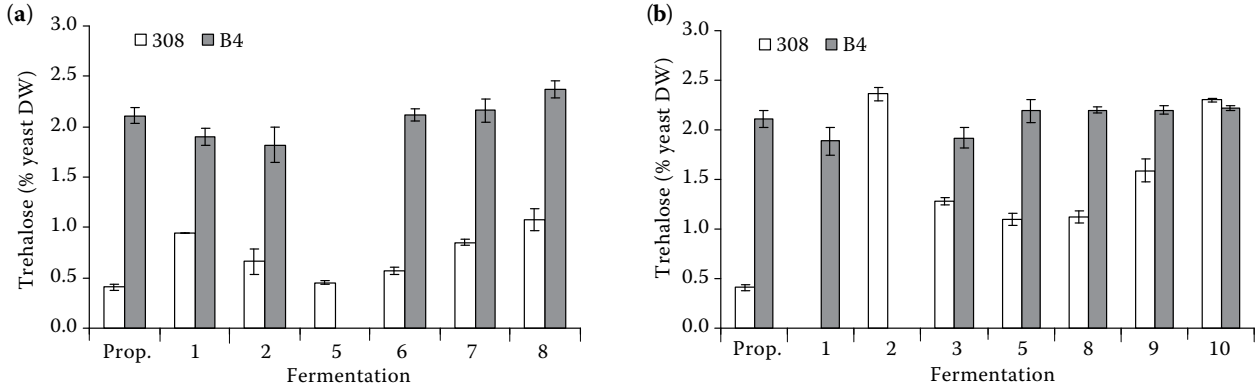


Figure 2. Trehalose content in yeast cells of strains 308 and B4 after propagation and successive fermentation of 15°P (**a**) and 10°P (**b**) wort

### Trehalose content in repitched yeast

During serial repitching when 15°P wort was fermented, a decline in trehalose content was observed in strain 308 after initial fermentation processes (Figure 2a). Trehalose content increased in the last generations. All these changes were statistically significant at $P < 0.05$. The accumulation of trehalose in repeatedly used yeast was also seen for strain B4, but not all changes were statistically significant. The accumulation of trehalose with increased generation number is in line with observations of Jenkins *et al.* (2003). However, the initial decrease in the first generations was not previously reported.

The accumulation of trehalose in the last generations of both strains was also observed when 10°P wort was fermented (Figure 2b). The trehalose level remained invariable in the first generations of strain B4. Similar data were not obtained in the case of strain 308.

For strain 308 the trehalose level in cropped slurries was much higher than in the propagation sample (Figures 2). This tendency was not found for strain B4.

### Effect of serial repitching on yeast physiological condition

The impact of yeast exposure to repeated cycles of stress during its employment in successive fermentations on slurry quality was previously examined both for lager yeast (Jenkins *et al.* 2003; Kobayashi *et al.* 2007) and ale yeast (Smart & Whisker 1996; Powell & Diacetis 2007). In these investigations the effect of serial repitching on yeast flocculation and surface characteristics, viability, membrane integrity, acidification power test results, intracellular carbohydrate and isoamyl alcohol concentration was analysed. Despite a lot of studies related to this issue, the influence of extended reusing of the same yeast on yeast quality, including physiological state, has not been well elucidated.

In Jenkins *et al.* (2003) study the evaluation of glycogen and trehalose content was conducted on seven sequentially harvested yeast samples. The progressive accumulation of trehalose in successive yeast generations and the constant level of glycogen starting from the 4$^{th}$ generation were observed. In our investigation an increase in trehalose content

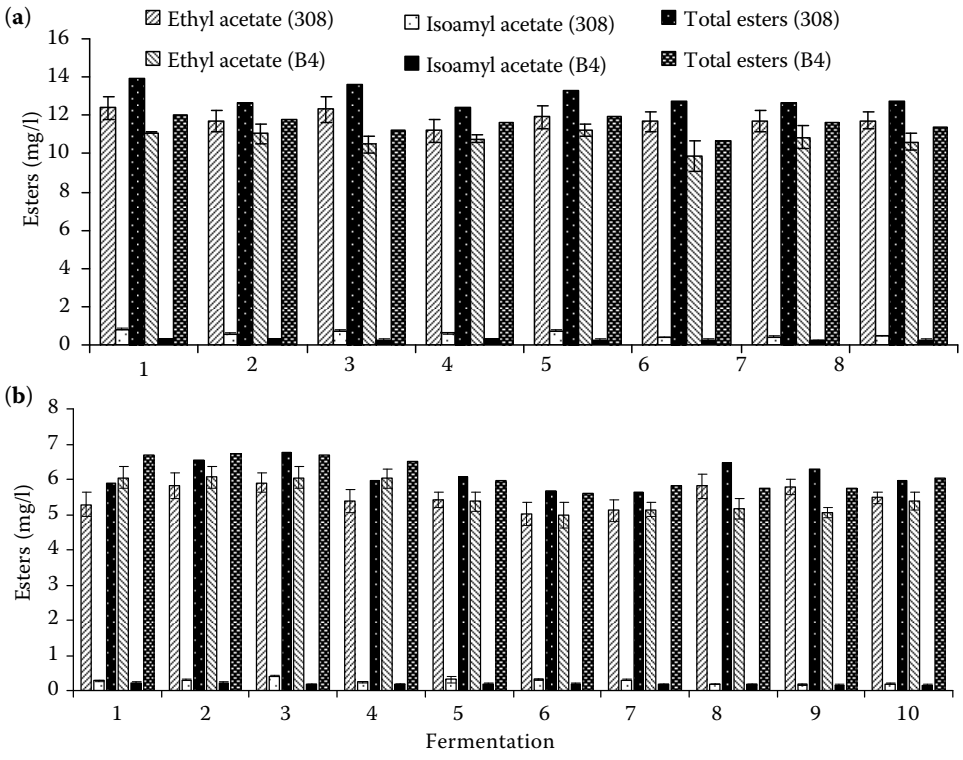


Figure 3. Effect of yeast generation number on ester content in green beer produced from 15°P (**a**) and 10°P (**b**) wort with strain 308 and B4

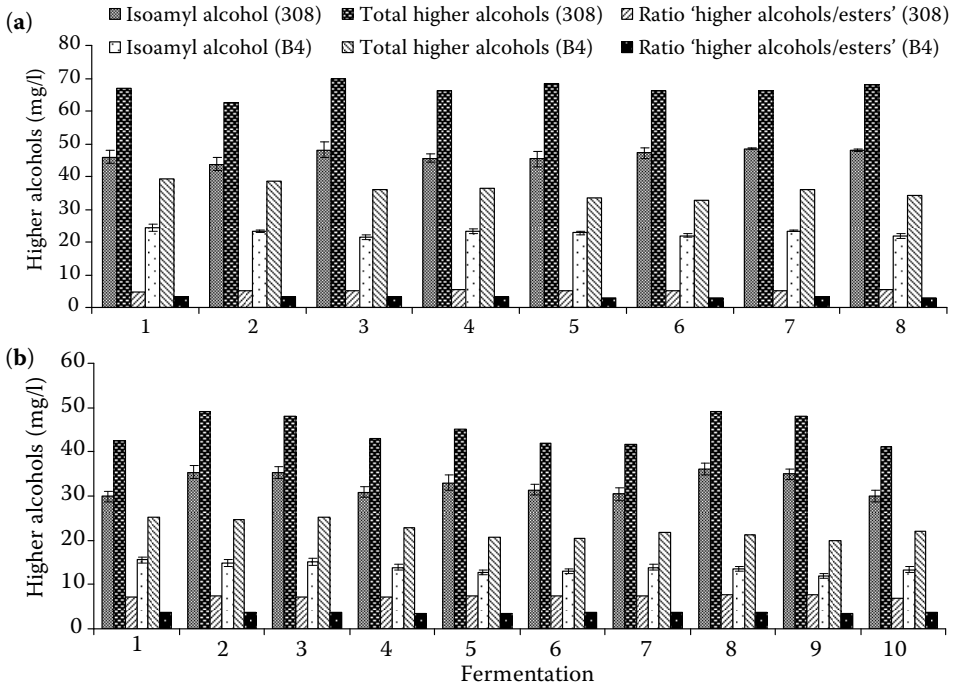


Figure 4. Effect of yeast generation number on higher alcohol content in green beer produced from 15°P (**a**) and 10°P (**b**) wort with strain 308 and B4

and a simultaneous decline in glycogen content in aged culture were found out. However, a decline in the glycogen level was observed only for the old culture of the last evaluated generations. Jenkins *et al*. (2003) did not report the original gravity of wort used in their study and other fermentation conditions and therefore a comparison of the results has to be conducted with caution.

The number of serial repitchings which did not cause any change in intracellular carbohydrate content depended on original wort gravity to a large extent. A drop in the glycogen level during fermentation of 15°P wort was observed earlier than during fermentation of 10°P wort. It was connected with higher osmotic stress and ethanol concentration generated when higher gravity wort was fermented. It implies a faster impairment of the yeast physiological state during fermentation of higher gravity wort.

Kobayashi *et al*. (2007) also characterised the physiological state of recycled lager yeast by determination of membrane potential and isoamyl alcohol secretion. However, in their work yeast was not used to ferment normal brewery wort but yeast extract-dextrose medium, which only imitated industrial low-malt wort. They observed the most distinct changes in the growth rate and isoamyl alcohol production and stated that yeast harvested after the fourth fermentation should not be reused any longer. In our study no significant variation in the flavour of beer produced with successive yeast generations was noticed (Figures 3 and 4). In turn, a change in glycogen and trehalose content in yeast was found out. But the decline in glycogen content was not seen sooner than in the $7^{th}$ generation. In yeast used for fermentation of 10°P wort, alterations occurred even in the ninth generation. Yeast employed in our study was much more resistant to repeated exposure to stress. However, in our investigation apart from different medium, different fermentation temperature was also applied. Fermentations were performed at 9°C instead of at 15°C as it was in Kobayashi *et al*. (2007) work. And the temperature of fermentation as well as fermentation medium have a considerable influence on yeast quality and production of flavour compounds.

Quilter *et al*. (2003) repitched the same yeast seven times in succession and noted that aged yeast cultures produced higher quantities of esters such as ethyl acetate and isoamyl acetate. With each repitching the amount of isoamyl acetate increased steadily until the $5^{th}$ repitching, after which it began to fall. In our investigation the initial increase in isoamyl acetate secretion was not observed (Figure 3). But after the seventh repitching a slight decrease was observed for strain 308 during fermentation of 10°P wort and after

the fifth for 15°P wort. Ethyl acetate synthesis was not a generation number dependent. In general, there was not any tendency for the level of esters to rise with the yeast generation number. Both Quilter *et al*. (2003) and Kobayashi *et al*. (2007) demonstrated that the number of serial repitchings might affect the secretion of higher alcohols by yeast. Quilter *et al*. (2003) found out that the amount of amyl alcohol increased steadily up with each repitching until the 5$^{th}$ repitching, after which it began to fall. These phenomena were not observed in our research. Multiple yeast reusing did not affect the total amount of higher alcohols (Figure 4). Furthermore, no difference in the 'higher alcohols to esters' ratio was noticed either. The yeast ability to produce a correct ratio of flavour compounds remained stable.

Breweries have to carefully consider how many fermentations to pitch with the same yeast cultures. Since the yeast physiological condition and consequently beer quality depend on many factors, such as strain, wort gravity, yeast handling procedure, it is difficult to settle in general how many times to reuse yeast in the brewery. In particular, because researches are conducted in different conditions and contradictory findings are often reported. This study emphasised that there is no reason for an extreme reduction of the number of yeast repitchings without controlling the individual strain susceptibility to extended reusing.

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Received for publication February 28, 2012
Accepted after corrections September 4, 2013

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