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REVIEW ARTICLE

Serial re-pitching: its effect on yeast physiology, fermentation performance, and product quality

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Abstract

Background Serial re-pitching is a term given to a practice whereby yeast harvested at the end of fermentation is re-used in subsequent fermentations.

Purpose The purpose of this paper was to review and summarize existing literatures, research data, and case studies to illustrate the effect of re-pitching on the physiology and fermentation performance of brewing yeast and the resulting quality of beer.

Methods Data related to biomarkers used to assess yeast physiology and fermentation performance and quality of beer were compared for various articles.

Results And comparison of the results was done with caution as many of the studies were conducted using different yeast strains, wort gravity, pitching rate, and other fermentation conditions.

Conclusion This study confirms that serial re-pitchings aggravate the effect of pitching rate, wort gravity, cell age, yeast oxygenation, and yeast strain on yeast cell physiology, fermentation performance, and quality of final beer. However, further empirical research at molecular level is crucial.

Keywords Flocculation · Flavor compounds · Physiology · Serial re-pitching · Viability · Vitality

Introduction

At the end of brewing fermentation, yeast biomass is cropped from the bioreactor, maintained in refrigeration, and re-inoculated into a fresh batch of wort several times. Since yeast propagation is time and resource intensive, it is customary to serially re-pitch yeast for considerable times prior to propagation. Serially re-pitching, whereby yeast biomass harvested upon completion of fermentation is re-used in consecutive fermentations, is of a subject of study.

The number of yeast re-pitching varies among breweries. Some breweries use a lager yeast culture up to 20 times of the same fermentation conditions and wort gravity (Stewart 2009; Kordialik-Bogacka and Diowksz 2013; Bühligen et al. 2013). Yeast cultures in typical brewery fermentation divide nearly two to three times times (Powell et al. 2003a). In light of this, it would represent nearly 13–25 serial re-pitchings (Powell and Diacetis 2007). However, re-using the same batch of yeast makes the brewing process even more difficult to control (Bleoancă and Borda 2013).

During fermentation, brewing yeasts are exposed to temporal changes in oxygen concentration, osmotic potential, low pH, ethanol concentration, and carbon and nutrient limitation as well as temperature shift (Trevisol et al. 2011; Meledina et al. 2015). Fermentation performance of brewing yeast is dependent on its ability to adapt to these environmental stresses, especially during serial re-pitching of the same yeast culture over a number of fermentations (Gibson et al. 2007).

Following pitching, the yeast grows exponentially after a brief lag phase, rapidly depleting the available oxygen and creating an anaerobic environment. The wort sugars and assimilable nutrients are also quickly consumed resulting in carbon and nutrient deficit, allowing the cell to remain in a dormant state, a common response to nutrient depletion in yeast cells (Gibson et al. 2007).

Prior to re-pitching, the cropped yeast is stored in cold water under starvation and anaerobic conditions. Thus, yeast is predominantly exposed to anaerobic environments in the brewery (Gibson et al. 2007). Clearly speaking, having been recovered from a previous fermentation, pitching yeast is in

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stationary phase (Bolat 2008). During re-pitching, yeast biomass is inoculated into aerated wort. It is therefore apparent that yeast cell is subjected to a sudden change from anaerobic to aerobic (Kobi et al. 2004; Kuřec et al. 2009a), an osmotic pressure (Pratt et al. 2003), and starvation to nutrient-rich conditions to which the yeast has to adapt quickly in order to carry out a desirable fermentation performance (Kuřec et al. 2009a).

The effect of yeast exposure to repeated cycles of stress during fermentations on yeast slurry quality has been reported both for bottom-fermenting yeast (Kobayashi et al. 2007; Gabriel et al. 2008; Verbelen et al. 2009b; Kordialik-Bogacka and Diowksz 2013; Bühligen et al. 2013) and top-fermenting yeast (Kobi et al. 2004; Powell and Diacetis 2007). Several but contrary results have been reported. Whether the number of yeast generation may have an impact on yeast cell physiology, fermentation performance, and the resulting brewing quality is still a subject controversy.

The scope of this review comprises a reflection on how re-using yeast culture could influence yeast physiological and fermentation performance using specific biomarkers as indicator in subsequent generations. Moreover, information on flavor contributor compounds in the final beer was also addressed to provide insight on the relationships between serial re-pitching and final product.

Yeast physiological markers

Effect of re-pitching on yeast physiological state

Serial re-pitching subjects a yeast culture to recurrent stress that may initiate reversible or irreversible injury, depending on the strain (Layfield and Sheppard 2015). Levated osmotic pressure, increased alcohol concentration, and modified nutrient balance may distort yeast metabolism (Pratt et al. 2003; Kordialik-Bogacka and Diowksz 2013).

A number of yeast biomarkers exist, each reflecting activity in at least one physiological system. Contents of intracellular glycogen, trehalose, sterol and non-saturated fatty acids, bud scars, DNA, and intracellular proteinases are the commonest biomarkers used to monitor and predict physiological activities of yeast cells (Verbelen et al. 2009b; Kordialik-Bogacka and Diowksz 2013; Meledina et al. 2015). After several re-pitchings, aged yeast cells can result in leakage of intracellular compounds into the fermented liquor (Wang et al. 2019). Contents of intracellular glycogen and trehalose which are the source of endogenous glucose used during the log growth phase vary in response to changes in physiological condition (Bolat 2008; Meledina et al. 2015). Those physiological states of viable cells determine yeast performance in alcoholic fermentation (Bouix and Leveau 2001). Thus, it is possible to ascertain yeast physiology based on assaying the level of glycogen, trehalose, and bud scars. Of the commonly used physiological parameters, assessment of yeast viability and vitality was also considered in this review.

Cell count in re-pitched yeast

A decreasing and/or no trend of cell growth associated with re-pitching have been reported. Verbelen et al. (2009b) noted that HCD (pitching rate, 80×10^6 cells/mL) with aerated wort and non-pre-oxygenated yeast (A/NP) condition showed a decreasing trend of net growth and reached a minimum in G4. The drop observed in growth was also accompanied by a drop in the UFA/SFA index, an indicator for the membrane fluidity, which is of importance for yeast growth. Consequently, the A/NP yeast slurries were quantitatively and qualitatively incapable of being re-pitched for 7th generation, due to repeated small losses associated with cropping and pitching along the previous conducted fermentations.

Sigler et al. (2009) performed three generations of fermentations in 12°/12°/12°, 16°/16°/12°, and 20°/20°/12° worts. In the 3rd pitching at 12°P wort fermentation, the cell count dropped by 10%. In 16°P wort fermentations, the cell count dropped by 12% in the 2nd pitching compared with the 1st pitching. Likewise, re-pitching in 20° wort caused the decrease in cell count (18%) in the 2nd pitching. The 3rd "back to normal" re-pitching into 12° wort increased the cell count again to the level observed in the 1st pitching into 16 and 20° worts, suggesting the profound effect of wort gravity aggravated by re-pitching. In line with this report, Kobayashi et al. (2007) reported decreasing trend of the specific growth rate and optical density (660) after multiple re-pitchings.

Adequate yeast growth has to be assured to produce a sufficient amount of cells capable of re-pitching at the same inoculum size as the previous fermentation (Verbelen et al. 2009b). To improve the cell growth in HCD and REF fermentations, yeast was pre-oxygenated and this improved sufficient formation of UFA, which in turn, results enhanced net growth of yeast.

Cell viability and vitality in re-pitched yeast

The viability and vitality of the yeast are main concern to the brewers. Yeast viability is explained by the ability of cells to grow and reproduce themselves (Magalhães et al. 2011) which discriminates between dead and live cells (Bleoancă and Borda 2013). Using viability tests in brewing industry, a correction factor can be established to assure the same concentration of viable cells for all fermentation batches. Unluckily, viability tests are unable to predict the ratio between the total number of cells and the viable and/or dead cells. In these conditions, vitality assays are promising alternative, being capable of showing the physiological state of the brewing yeast cells (Bleoancă and Borda 2013).

Table 1 Methods of assessing brewing yeast viability and vitality

Methods	Types	Output	Measure	Sources
Methylene blue staining	Manual	Stains dead cells	Viability	Kuřec et al. 2009a; Stewart 2009; Magalhães et al. 2011; Kordialik-Bogacka and Diowksz 2013; Layfield and Sheppard 2015; Meledina et al. 2015
Mg-ANS	Manual	Dead cells have fluorescent luminescence	Viability	Meledina et al. 2015
Dihydrorhodamine staining		Stains dead and physiologically poor cells	Viability and vitality	Meledina et al. 2015
Methylene blue with safranin O staining	Manual	Stains dead, weaken, and active cells	Viability and vitality	Meledina et al. 2015
Acidification power test (AP)	Automated	PH drop 10 min before and after glucose addition	Vitality	Gabriel et al. 2008; Kuřec et al. 2009a; Sigler et al. 2009; Meledina et al. 2015
Vitaltitration method	Automated	pH drop in time (from pH 10.0 to pH 6.5)	Vitality	Magalhães et al. 2011; Bleoancă and Borda 2013
Flow cytometry	Automated	Viability: count of fluorescent yeast; vitality: index of ΔFI_{15}	Viability and vitality	Bouix and Leveau 2001; Ginovart et al. 2011
Measurement of NAD(P)H	Automated	Change of NAD(P)H fluorescence intensity at 340/440 nm	Vitality	Kuřec et al. 2009a; Magalhães et al. 2011

Mg-ANS, magnesium salt 1-anilino-8-naftalen of sulfonic acid; *NAD(P)H*, reduced form of nicotinamide adenine dinucleotide (phosphate)

Yeast vitality refers to a group of characteristics of microbes related to their metabolic competence and can be perceived as their ability to respond to or handle stress situations occurring during pitching (Kuřec et al. 2009a, b), propagation, serial pitching, yeast cropping, and storage (Gabriel et al. 2008). Having the same numbers of "healthy active yeast" ready to ferment batch after is the main challenge in brewing. Evaluating yeast vitality is necessary so that corrective measure can be taken prior to re-pitching in order to optimizing the quality of fermentation (Bouix and Leveau 2001). Methods for assessing yeast vitality have been described (Table 1). Although the physical and fermentative characteristics of the yeast culture may appear consistent, yeast cells can degenerate (Kuřec et al. 2009a) and genetic mutations can occur during yeast recycling (Powell and Diacetis 2007).

Kordialik-Bogacka and Diowksz (2013) observed that the viability of yeast cells did not change significantly after ten consecutive fermentations. This report was in disagreement with previous studies (Kobayashi et al. 2007; Gabriel et al. 2008; Sigler et al. 2009). In Kobayashi et al. (2007) study, the fluorescence intensities of fluorescent dyes dihydrorhodamine 123(DHR), an indicator of reactive oxygen species (ROSs), and bis-(1,3-dibutylbarbituric acid) trimethine oxonol (OXN), which indicates membrane potential, exhibited progressive increase as the number of serial re-pitching cycles increased. Accumulation of reactive oxygen species (ROS) in the yeast cell causes cell damage, leading to a decline in cell vitality and viability (Wang et al. 2019). In both cases (DHR and OXN), the cells are in poor physiological condition. This report was supported by Gabriel et al. (2008), who detected that AP value decreased with successive re-pitching, notifying that the yeast vitality decreased with increasing number of re-pitchings.

Similarly, Sigler et al. (2009) noted that yeast vitality (measured as AP, acidification power) was reduced up to 10% with repeated cycle of re-pitching. In this study, the higher AP reduction observed on re-pitching in conventional HGB worts was explained in terms of the toxicity of increased ethanol levels produced under these conditions. Measurement of pH in yeast slurries, for re-pitching, can be an indication of yeast viability and yeast autolysis. Besides, it can be used as a tool to monitor yeast fitness during an ongoing fermentation. Wort acidification, or pH downshift, occurs as a result of the consumption of wort carbohydrates and buffering compounds and the production of carbonic acid (from CO_2 generation) and some organic acids (Layfield and Sheppard 2015).

The viability in REF (NCD) condition dropped in the first two subsequent fermentations and after 6th generation in the A/NP condition while it remained high in the A/PR condition (Verbelen et al. 2009b), thus showing the impact of exposure of cells to a repeated cycle of stress associated with oxygen deficit both in the REF and A/NP conditions.

Cell age in re-pitched yeast

Cell age in budding yeast is defined by the number of times an individual cell has divided, termed as replicative lifespan/aging (Layfield and Sheppard 2015). When cells age, they are subjected to typical cell surface changes. Population of a given culture is composed of individual cells of varying age. Daughter cells exhibit a frail structure called birth scar which is distinct due to their large size, while mother cells display a more persistent bud scar (Powell et al. 2003b). The number of bud scars present on the cell surface is directly correlated to the number of divisions an individual cell has undertaken (Kuřec et al. 2009b; Layfield and Sheppard 2015), thus represents a biomarker for replicative cell age estimation (Kuřec et al. 2009b; Verbelen et al. 2009b) and can be determined using confocal microscopy (Powell et al. 2003b; Kuřec et al. 2009b) (Table 2).

Aged yeast cells are often associated with decreased fermentation capacity, changed morphology, altered flocculation patterns, and extended generation times (Powell et al. 2003a). A strong increase of old cell in HCD of the A/NP condition and a less steep increase in the A/PR condition during serial re-pitching were reported (Verbelen et al. 2009a). Conversely, there was no progressive aging detected during serial re-pitching (Bühligen et al. 2013).

Glycogen content in re-pitched yeast

Glycogen is the major storage polysaccharide in brewing yeast cells, accounting for up to 40% of the dry weight of cells (Schlee et al. 2006; Gibson et al. 2007; Bolat 2008). This intracellular carbohydrate is essential as an energy source for cell activity since wort sugars are not assimilated early in the lag phase of fermentation (Bolat 2008). It provides energy and carbohydrates for the synthesis of sterols and lipids during the aerobic phase of fermentation (Schlee et al. 2006; Bolat 2008).

Accumulation of glycogen starts in the early exponential growth phase and continues when nutrients are available until the yeast batch enters the stationary phase (Gibson et al. 2007; Bolat 2008; Stewart 2009). Brewing yeast metabolizes the stored glycogen into glucose at times of nutrient limitation. When the yeast is pitched, glycogen reserves are catabolized for sterol synthesis. Besides, brewing yeast catabolizes the stored glycogen into glucose at the end of fermentation (Schlee et al. 2006) which is utilized by the cell for reproduction and cell maintenance during dormancy (Bolat 2008). Thus, its concentration often determines yeast growth and yeast fitness.

Kordialik-Bogacka and Diowksz (2013) investigated gradual decline of glycogen content in successive generations of *Saccharomyces pastorianus*. Yet, the reduction in glycogen content of yeast is influenced by wort gravity, causing osmotic stress and ethanol concentration, to a large extent than the number of serial re-pitchings. Earlier, Verbelen et al. (2009b) reported no trend in glycogen content during serial re-pitching of eight consecutively harvested yeast cells. However, a drop in glycogen was observed at G5 in the A/PR condition, coupled with increase in UFA formation and yeast growth. The level of intracellular glycogen must be maintained for sustaining the viability and vitality of yeast which in turn ensures that the lag phase is kept to a minimum when the yeast is pitched into wort (Stewart et al. 2013).

Intracellular trehalose content in re-pitched yeast cell

Trehalose is a non-reducing disaccharide of glucose units linked in an α–1, 1-glycosidic linkage. It makes up 23% or more of the dry weight of the *Saccharomyces cerevisiae* (Bolate, 2008). It serves as a carbon source during the initiation of the cell cycle (Kordialik-Bogacka and Diowksz 2013) and nutrient-depleted conditions. More importantly, it assists as a stabilizer and protectant of cellular membrane (Bolat 2008) by substituting water and binding to the polar head groups of phospholipids (Trevisol et al. 2011). It acts as a stress protectant (Gibson et al. 2007; Zhimin et al. 2012) and fortifies the yeast cell wall (Schlee et al. 2006). It is required for improving fermentative capacity, extends life span, and reduces petite generation (respiratory deficient cells) as well as their ability to withstand stressful industrial conditions (Trevisol et al. 2011).

Trehalose accumulation is associated with stress factors induced by low water activity, high ethanol, osmotic pressure, freezing and high temperature (Zhimin et al. 2012; Kordialik-Bogacka and Diowksz 2013), oxygenation and carbonation

Table 2 Methods of detecting intracellular carbohydrate and bud scars of yeast cell

Biomarkers	Methods	Wave length	Output
Glycogen	Acriflavine staining	488 nm	Emits green fluorescence
Trehalose	Lectin-fluorochrome-conjugate concanavalin A–fluorescein	488 nm	Detects green fluorescence
Bud scars	Staining with WGA fluorescein dye	500–580 nm	Circular, bright yellow-green fluorescent on the cell surface: high fluorescent-many aged cells; low fluorescence- a young cell in the population

Schlee et al. 2006; *WGA*, wheat germ agglutinin fluorescein

(Trevisol et al. 2011), growth restriction, and nitrogen limitation (Gibson et al. 2007; Zhimin et al. 2012). It is involved in the protection of yeast cells against reactive oxygen species (ROS) (Gibson et al. 2007) associated with respiration. It is a key stress indicator in brewing yeast cultures (Zhimin et al. 2012; Kordialik-Bogacka and Diowksz 2013; Meledina et al. 2015) and a general response to protect the cell against stress (Stewart 2009). Therefore, level of intracellular trehalose is used as a biomarker of physiological state of yeast cells.

In this regard, an increase in trehalose content of ten consecutively harvested yeast generations was reported (Kordialik-Bogacka and Diowksz 2013), suggesting serial re-pitching results in cycle of stress and associated response, initiating trehalose buildup. The higher trehalose levels seen in the non-pre-oxygenated yeast condition, compared with pre-oxygenated yeast, indicated that this population experienced more stress, possibly associated with the progressive loss of viability during re-pitchings (Verbelen et al. (2009b). Increasing trehalose content during serial re-pitching is inconsistent with previous reports (Gibson et al. 2007; Stewart 2009; Trevisol et al. 2011).

Initial decline in trehalose contents of the first yeast generations was reported during serial re-pitching at 15°P wort fermentation (Kordialik-Bogacka and Diowksz 2013). This report is in line with the findings of Verbelen et al. (2009b), who observed a decreasing trend of trehalose content during the first 4–5 generations. This indicates that as the cAMP/PKA pathway is active during each initial phase of fermentation, trehalose is broken down every time it is re-pitched in glucose-rich wort (Verbelen et al. 2009b). Apparently, mutant strains of *S. cerevisiae* having defects in trehalose metabolism maintained the highest levels of trehalose after 24 h of fermentation and showed the highest survival rates upon re-pitching (Trevisol et al. 2011).

Trevisol et al. (2011) reported progressive to complete exhaustion in trehalose reserves at the end of the first fermentation cycle. The decline in trehalose level is consistent with Stewart (2009). As the yeast adapted the stress conditions imposed by the wort and storage-linked stress cycle, the trehalose levels decreased (Gibson et al. 2007; Stewart 2009; Verbelen et al. 2009b). Verbelen et al. (2009c) provide evidence that pre-oxygenating yeast cells appear to attain stress resistance by accumulating the stress-protectant trehalose and induce the expression of genes involved in detoxification processes and stress-responsive genes.

Effect of serial re-pitching on fermentation performance

Fermentation performance of yeast biomass can be evaluated in terms of flocculation capacity, attenuation, and fermentation time.

Attenuation

Attenuation refers to the extent to which the yeast converts wort to alcohol and CO_2. It is a key parameter for determining the fermentation performance of brewing yeast cells. No significant difference was reported in terms of attenuation profiles during re-pitchings of ale yeast at varying wort gravity (10°P and 15°P) (Kordialik-Bogacka and Diowksz 2013). Similarly, serial re-pitching up to 13 times did not affect the apparent degree of fermentation (ADF) (Speers and Stokes 2009). This study was supported by previous report (Powell and Diacetis 2007). Contrarily, Sigler et al. (2009) observed slowing down of wort attenuation during successive re-pitchings in 20° wort. Apparently, the wort attenuation rate in the 2nd pitching dropped and fermentation was stopped after 84 and 156 h. Thus, re-pitching caused a progressive slowing down of fermentation rate, a 45% drop in the 2nd and 54% drop in the 3rd pitching. In the same fashion, a decreasing trend in apparent degree of attenuation was reported during serial re-pitches (expressed as in terms T1/2-AP relationship) (Gabriel et al. 2008).

Effect of serial re-pitching on fermentation time

Sigler et al. (2009) noticed 86%, 65%, and 39% delay of fermentation time in the 2nd pitching in 20°, 16°, and 12° worts, respectively. Similarly, a marked (39%) slowing down of fermentation was also found when 3rd yeast generations of all treatment groups were pitched in 12° wort. This implies the delay in fermentation time is largely associated with serial re-pitching than wort gravity. However, the 3rd "back to normal" pitching into 12° wort caused a substantial shortening of the fermentation time relative to the 2nd pitching, revealing that the effect of wort gravity is in place (Sigler et al. 2009).

Gabriel et al. (2008) exhibited that the fermentation time increased with increasing number of generation. Similarly, Sigler et al. (2009) observed that re-pitching caused a progressive slowing down of fermentation time, the 4.0% alcohol level being reached after 65, 94, and 100 h in the 1st, 2nd, and 3rd pitching, respectively. The prolonged fermentation times of REF in G1 and G2 could explain the observed drop in viability (Verbelen et al. 2009b). Extended fermentation time in NCD yeast recycled for the 2nd and 3rd time was reported by Verbelen et al. (2009b), thus having a direct financial implication to the brewing industry.

Effect of serial re-pitching on flocculation capacity

Flocculation is a crucial step in the downstream process of brewing. The term "flocculation" applies to "the phenomenon wherein yeast cells adhere in the form of large cell clumps (flocs) and either sediment from or rise to the medium's surface." It is pre-condition for mass sedimentation of yeast

during brewing (Vidgren and Londesborough 2011). Thus, the yeast biomass can be cropped by sedimentation from the bottom (lager strain) or by flotation from the top (ale strain), because of cell aggregates entrapping CO_2 bubbles, of the fermenter and re-pitched in subsequent fermentation (Gibson et al. 2007; Kuřec et al. 2009b; Stewart 2009).

The timing of onset of flocculation is essential (Gibson et al. 2007; Vidgren and Londesborough 2011) where there is commercial interest (Stewart 2009); yeast biomass is removed earlier to decrease process time via a "warm" or "early" cropping regime (Powell et al. 2003a; Kuřec et al. 2009b; Ginovart et al. 2011). Early flocculation, before the wort is completely attenuated, is not desirable, as it causes sluggish or stuck fermentation and final beers with high residual sugars and unsatisfactory flavor characteristics. Rather, strong and nearly complete flocculation at the end of the fermentation is ideal, providing a cheap, effective, and eco-friendly way to remove nearly all yeast cells from the green beer (Vidgren and Londesborough 2011).

Brewing yeast shows undesirable changes in its flocculation characteristics during serial re-pitching (Sato et al. 2001; Vidgren and Londesborough 2011). The role of flocculation is a means to harvest a yeast biomass at the end of fermentation, so that it can be re-pitched in a subsequent fermentation. The ideal brewing yeast exhibits constant flocculation during successive run of fermentation, cropping, and re-pitching (Vidgren and Londesborough 2011).

Sigler et al. (2009) noted that re-pitching dramatically reduced yeast sedimentation ability and delayed the onset of the sedimentation. In their study, second-generation yeast biomass was characterized by a strong drop in yeast sedimentation capacity in all three variants (12°, 16°, and 20° worts). The main factor affecting yeast sedimentation was therefore likely to be the number of re-pitchings, not wort osmolality. This report is in line with previous report (Sato et al. 2001). Variability of flocculation in bottom-fermenting yeast was explained by genetic alterations, such as loss in the flocculation gene Lg-*FLO1* which governs flocculation characteristics. This was observed and might occur frequently in brewing yeasts through successive generations. Conversely, no significant variation in flocculation characteristics of the yeast biomass was detected during serial re-pitching (Powell and Diacetis 2007; Bühligen et al. 2013). Powell and Diacetis (2007) proposed that the lack of variability between each lager populations may reflect the unique re-pitching regime employed during the recycling of this strain which does not facilitate selection for specific individuals.

Successive cropping and re-pitching with a specific portion of the cone may lead to a drift in the characteristics of the cropped yeast. Highly flocculent cells were found to be preferentially located in the middle layer of the cone (Vidgren and Londesborough 2011). Thus, it is crucial to collect, wash, store, and re-use the potentially most productive segment of the settled yeast biomass (Kuřec et al. 2009b). Yeast flocculation is also influenced by the replicative age of the yeast population (Gibson et al. 2007; Layfield and Sheppard 2015). Typically, the yeast cropped from the fermentation vessel to be used for serial re-pitching is the center-top portion, theoretically comprising middle-aged and virgin cells (Kuřec et al. 2009b; Layfield and Sheppard 2015).

Effect of serial re-pitching on product quality

Flavor compounds/volatiles

The brewing yeast produces a number of aroma-active low-molecular-weight secondary metabolites which contribute to the flavor of alcoholic beverages (Saerens et al. 2008; Magalhães et al. 2011; Pires et al. 2014). Flavor compounds build a sensory profile specific to a brewing brand, therefore, influences beer quality. They are intermediates or by-products of yeast in pathways leading from the catabolism of wort components (sugars, nitrogenous compounds, and sulfur compounds) to the synthesis of components required for yeast growth (Lodolo et al. 2008; Pires et al. 2014).

These include higher/fusel alcohols, esters, carbonyls (aldehydes/ketones), vicinal diketones (VDK) (diacetyl and pentanedione), fatty and organic acids, and sulfur compound (Lodolo et al. 2008). They impart a considerable impact on the overall organoleptic characteristics of the final product (Magalhães et al. 2011). Higher alcohols and esters are desirable volatile constituents of a pleasant beer while VDKs are often considered as off-flavors. Similarly, yeast metabolism contributes organic acids, sulfur compounds, and aldehydes (Pires et al. 2014).

Higher alcohols include n-propanol (alcohol sweet flavor, threshold 600 mg L^{-1}), isobutanol (solvent, threshold 100 mg L^{-1}), isoamyl alcohol (alcoholic banana flavor, threshold 50–65 mg L^{-1}), amyl alcohol (alcoholic solvent flavor, threshold 50–70 mg L^{-1}), and 2-phenylethanol (rose, threshold 40 mg L^{-1}) (Pires et al. 2014).

Esters, which provide fruity and flowery flavors to beers, are the most important aroma elements produced by yeast. Acetate esters include ethyl acetate (a solvent-like flavor), isoamyl acetate (banana-like flavor), and ethyl caproate (apple-like flavor). Ethyl esters include ethyl hexanoate (sweet apple-like aroma, threshold 0.2 mg L^{-1}), ethyl octanoate (sour apple aroma, threshold 0.9 mg L^{-1}), and ethyl decanoate (floral odor) (Saerens et al. 2008; Pires et al. 2014).

Carbonyl compounds comprise acetaldehyde and diacetyl (2,3-butanedione) and 2,3-pentanedione. Organic acids impart a "sour" flavor to beers. These include pyruvate, acetate, lactate, citrate, succinate, and malate. They are formed at relatively low levels and small change in their concentrations can have significant effect on the final sensorial quality of fermented beverages (Saerens et al. 2008).

The secretion and level of these compounds could be influenced by physiological condition and the overall metabolic balance of the yeast (Magalhães et al. 2011; Kordialik-Bogacka and Diowksz 2013). The good yeast quality is precondition to get consistent beer with correct flavor balance (Kordialik-Bogacka and Diowksz 2013).

The influence of re-pitching on the concentrations of flavor volatile esters, higher alcohols, carbonyl compounds, and organic acids was retrieved from literatures. Kordialik-Bogacka and Diowksz (2013) noticed no significant variation in the flavor of beer produced with successive yeast generations. This report was supported by previous findings (Powell and Diacetis 2007; Speers and Stokes 2009; Verbelen et al. 2009b). However, these findings are inconsistent with earlier studies (Kobayashi et al. 2007; Quilter et al. 2003). Yet other researchers come up with slightly different research outcome (Sigler et al. 2009; Verbelen et al. 2009a, 2009b).

Multiple yeast re-pitching did not affect the total amount of higher alcohols (Kordialik-Bogacka and Diowksz 2013). Likewise, Sigler et al. (2009) observed that the levels of propanol, isobutanol, and 2-methylbutanol exhibited no distinct trend with increasing number of re-pitching. Conversely, Quilter et al. (2003) found out an increasing trend of amyl alcohol with subsequent fermentation until the 5th re-pitching; after which, it began to fall. This finding was supported by Kobayashi et al. (2007), who observed an increase in isoamyl alcohol after multiple re-pitchings which causes an undesirable flavor. This lack of consistency with the Kordialik-Bogacka and Diowksz (2013) work was explained in terms of difference in fermentation medium and temperature. Thus, yeast employed in the Kordialik-Bogacka and Diowksz (2013) study was much more resistant to repeated exposure to stress, suggesting the temperature of fermentation and fermentation medium have a considerable influence on yeast quality and production of flavor compounds.

In the Kordialik-Bogacka and Diowksz (2013) study, the level of esters was stable in subsequent yeast generation. The stability of the levels of esters irrespective of serial re-pitching is supported by previous study (Sigler et al. 2009). Contrarily, Quilter et al. (2003) reported a substantial increase in isoamyl acetate and ethyl acetate production by re-pitching the same yeast seven times. With each re-pitching, the level of isoamyl acetate increased steadily until the fifth re-pitching; after which, it began to fall (Quilter et al. 2003). The declining of isoamyl acetate after the 5th generation is in line with the work of Kordialik-Bogacka and Diowksz (2013) who observed a slight decrease in isoamyl acetate secretion after the seventh re-pitching for 10°P wort and after the fifth for 15°P wort. In line with this, the REF condition exhibited an average 24 and 14% higher ethyl acetate and 15 and 37% higher isoamyl acetate production than the A/PR and A/NP conditions respectively during successive fermentations (Verbelen et al. 2009a). This suggests that the effect is largely due to serial re-pitching than oxygen condition and pitching rate.

Verbelen et al. (2009b) noted that the content of acetaldehyde remained fairly steady for the A/PR and REF conditions during serial re-pitching, while the A/NP condition showed accumulation of acetaldehyde through consecutive fermentations. Conversely, in Sigler et al. (2009) study, the contents of acetaldehyde decreased with number of fermentation cycle. The levels of acetaldehyde after 2nd fermentation at increased osmolarity were reduced while the 3rd fermentation in 12° wort brought them back to normal, inferring the effect was largely due to osmolarity instead of re-pitching.

Serial re-pitching up to 13 times did not affect the content of diacetyl, 2,3-pentanedione (Speers and Stokes 2009). Paradoxically, in Sigler et al. (2009) study, the contents of diacetyl and pentanedione in the beer increased through the course of serial re-pitching with increasing wort osmolarity. Compared with the values found at subsequent fermentation in12° wort, the levels of diacetyl after G2 in 16° and 20° worts increased and remained high even after subsequent pitching into normal gravity 12° wort, indicating that wort gravity seems the main factor affecting the content of diacetyl in this study, yet re-pitching appears to aggravate the effect. This increasing trend of diacetyl is in line with Verbelen et al. (2009b).

Verbelen et al. (2009b) noticed a drastic increase of total diacetyl (diacetyl and its precursor α-acetolactate) in the reference beer (REF) and in HCD fermentation after two generations of re-pitching with the A/NP condition, followed by A/PR in each cycle. Comparatively, the total diacetyl level in NCD/REF fermentations increased after G2 but still significantly lower than the HCD conditions. This was explained by insufficient chemical decarboxylation resulting from high fermentation rate of the REF condition since third generation. In the same fashion, the main actors here are pitching rate, aeration, and pre-oxygenation, while the number of re-pitchings seems to be a contributor to the accumulation of this flavor volatile.

The contents of dimethylsulfide decreased with number of fermentation cycle (Sigler et al. 2009). The levels of dimethylsulfide after the 2nd fermentation at increased osmolarity declined while the 3rd fermentation in 12° wort brought them back to normal, inferring the effect was largely due to osmolarity instead of re-pitching.

With respect to polypeptide profiles on beer during three successive fermentations, the effect of yeast generation was notable (Vieira et al. 2012). In line with this, Kobayashi et al. (2007) also observed increase in residual free amino acid nitrogen (FAN) concentrations after multiple re-pitchings.

Discussion

The likelihood of accumulation of mutant cells increases (Powell and Diacetis 2007), owing to the selective pressures (Sato et al. 2001), during serial re-pitching. Gradual change in chromosomal make up of yeast strains associated with yeast handling and fermentation processes has been reported (Casey 1996). A study on Ty and δ DNA sequences analysis, an indication of changes occurring at the molecular level, noticed some changes to the macro-morphological characteristics of an ale strain over the course of 98 serial re-pitchings but no genomic variations or changes to fermentation characteristics were observed (Powell and Diacetis 2007).

Sustainability of yeast serial re-pitching practice, by estimating the mRNA expression levels of cellular marker molecules which are known to correlate with metabolism, hexose transport, aging processes, stress response mechanisms, and flocculation capability, on changes in cell physiology over 20 cycles of fermentation suggested that *S. pastorianus* can be repeatedly re-pitched without loss of prominent physiological characteristics (Bühligen et al. 2013). This study was supported by Powell and Diacetis (2007). Inversely, in a study on the physiological state of recycled lager yeast by determining the membrane potential and ROS status estimation, a marked decline in the growth rate and vitality followed by apparent change in flavor compounds after the fourth fermentation was noted (Kobayashi et al. 2007).

A study on the dynamics of the ale-brewing yeast proteome, involved in carbohydrate metabolism, methionine biosynthesis, and stress-response pathways, at the beginning and end of the first and the third yeast generations observed a significant change (Kobi et al. 2004). Alteration in glycogen and trehalose level in re-pitched yeast was reported (Kordialik-Bogacka and Diowksz 2013). Increasing trend in trehalose level of re-pitched yeast indicates that propagation triggers a stress response (Verbelen et al. 2009b; Kordialik-Bogacka and Diowksz 2013) as observed by stress-response proteins (Hsp26p, Ssa4p, and Pnc1p) which exhibited constitutive expression in subsequent generations of ale strain (Kobi et al. 2004). Care must be taken when yeast is used to a subsequent fermentation, as high levels of trehalose detected in the pitching yeast offer a signal that yeast was submitted to stress factors during storage and handling (Bolat 2008).

Verbelen et al. (2009b) observed longer fermentation times in G1 suggesting that freshly propagated yeast differs remarkably from yeast derived from previous fermentations. This extended fermentation times could in fact be a result of aerobic/anaerobic switch (Kobi et al. 2004). NCD inoculum and non-pre-oxygenated HCD experience more stress than pre-oxygenated HCD yeast as indicated by inadequate growth which caused inadequate yield of biomass to re-pitch, a reduction in viability and increase of yeast age, a dramatic increase of the unwanted flavor compounds, acetaldehyde and total diacetyl, during the sequence of fermentations (Verbelen et al. 2009b).

The number of times a culture can be re-pitched or backslopped depends on the physiological state and fermentation performance of yeast, which in turn, depends on various factors as strain, yeast handling and cropping procedure, wort gravity, pitching rate, and other external factors. It is also governed by its replicative aging, which may be determined by the sensitivity to oxidative stress (Gibson et al. 2007). Generally, the number of re-pitchings that can be employed is reduced with increasing wort gravity. Though strain-to-strain variation has been observed, often times, ale strains are more susceptible than lager strains to recurrent re-pitching in high gravity wort (> 16°Plato) (Stewart 2009).

To determine the number of times a culture can be re-pitched, the brewing fitness of yeast population has to be examined. The brewing fitness includes the percent of viable cells, the vitality of these cells, and the suitability of the culture to a particular brewing environment based on the chemical composition of the wort, oxygen availability, and fermentation temperature (Layfield and Sheppard 2015). For validating brewing yeast fitness before re-pitching, and to predict the fermentations as well as the final product, samples of cropped yeast can be taken and compared with the stock culture using different techniques. Application of automated viability and vitality tests prior to pitching can possibly give insight on how many times the same yeast culture can be re-pitched in breweries.

Changes to the fingerprint of a culture over time can indicate that the yeast has undergone genetic drift, which may affect the course of fermentation and the characteristics of the final product (Powell and Van Zandycke 2009). Besides, the use of individual-based modeling (IbM) simulators, like INDISIM-YEAST, could offer diverse and attractive protocols related to “re-pitching” of yeast in the brewing industry (Ginovart et al. 2011).

The reduction of vitality is directly related to poor fermentative performances of yeast, leading to variable beer quality of subsequent batches (Bleoancă and Borda 2013; Kordialik-Bogacka and Diowksz 2013). High vitality results in a fast fermentation with minimal undesired by-products, while low vitality triggers sluggish or poorly attenuating fermentation (Bleoancă and Borda 2013). Besides, aged yeast cultures are better for producing higher quantities of esters than young cultures (Quilter et al. 2003). Early generation yeast tends to be slow to perform, while later generation yeast tends to exhibit genetic changes, poor viability, and inconsistent flocculation and flavor profiles (Smart 2011).

The physiological state of yeast affects the intensity of propagation and biosynthesis of secondary metabolites which in turn determines the sensory profile of beer. Physiologically active yeast is capable to adsorb positively charged colloids, such as proteins and phenolic substances due to negative

charge of their surface (Meledina et al. 2015). Thus, the less the normal physiological conditions of yeast, the less they adsorb the haze coursing substances and as a result, more substances remain in beer that raises its turbidity. Thus, re-pitching can only be conducted a number of times before the yeast quality deteriorates and fermentation performance is compromised. However, there is no reason for an extreme reduction of the number of yeast re-pitchings.

Conclusion

This study confirms that serial re-pitchings aggravate the effect of pitching rate, wort gravity, cell age, yeast oxygenation, and yeast strain on cell physiology, fermentation performance, and quality of final beer. Therefore, yeast oxygenation appears to be promising to attain yeast with brewing fitness for re-pitching and ideal fermentation performance and the resultant beer quality. However, further empirical research at molecular level is recommended to assure sustainability of serial re-pitching.

Compliance with ethical standards

Conflict of interest The author declares that he has no conflict of interest.

Research involving human participants and/or animals N/A

Informed consent N/A

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