J Ind Microbiol Biotechnol (2011) 38:1133–1144
DOI 10.1007/s10295-011-0999-3

REVIEW

Very high gravity (VHG) ethanolic brewing and fermentation: a research update

Pradeep Puligundla · Daniela Smogrovicova · Vijaya Sarathi Reddy Obulam · Sanghoon Ko

Received: 10 March 2011 / Accepted: 7 June 2011 / Published online: 22 June 2011

Abstract There have been numerous developments in ethanol fermentation technology since the beginning of the new millennium as ethanol has become an immediate viable alternative to fast-depleting crude reserves as well as increasing concerns over environmental pollution. Nowadays, although most research efforts are focused on the conversion of cheap cellulosic substrates to ethanol, methods that are cost-competitive with gasoline production are still lacking. At the same time, the ethanol industry has engaged in implementing potential energy-saving, productivity and efficiency-maximizing technologies in existing production methods to become more viable. Very high gravity (VHG) fermentation is an emerging, versatile one among such technologies offering great savings in process water and energy requirements through fermentation of higher concentrations of sugar substrate and, therefore, increased final ethanol concentration in the medium. The technology also allows increased fermentation efficiency, without major alterations to existing facilities, by efficient utilization of fermentor space and elimination of known losses. This comprehensive research update on VHG technology is presented in two main sections, namely VHG brewing, wherein the effects of nutrients supplementation, yeast pitching rate, flavour compound synthesis and foam stability under increased wort gravities are discussed; and VHG bioethanol fermentation studies. In the latter section, aspects related to the role of osmoprotectants and nutrients in yeast stress reduction, substrates utilized/tested so far, including saccharide (glucose, sucrose, molasses, etc.) and starchy materials (wheat, corn, barley, oats, etc.), and mash viscosity issues in VHG bioethanol production are detailed. Thereafter, topics common to both areas such as process optimization studies, mutants and gene level studies, immobilized yeast applications, temperature effect, reserve carbohydrates profile in yeast, and economic aspects are discussed and future prospects are summarized.

Keywords Very high gravity (VHG) · Brewing · Ethanol fermentation · Nutrient supplementation · Starch substrates · Immobilization · Optimization studies

P. Puligundla · S. Ko
Department of Food Science and Technology, Sejong University, 98 Gunja-dong, Gwangjin-gu, Seoul 143-747, Korea

D. Smogrovicova
Department of Biochemical Technology, Institute of Biotechnology and Food Science, Slovak University of Technology, Radlinského 9, 81237 Bratislava, Slovak Republic

V. S. R. Obulam (✉)
Department of Biochemistry, Sri Venkateswara University, Tirupati 517502, India
e-mail: ovsreddy@yahoo.com

Introduction

In recent years, brewing and bioethanol industries have been more focused on implementing cost-cutting measures to remain profitable during the economic downturn. One such measure is the adoption of emerging very high gravity (VHG) fermentation technology owing to its process productivity-enhancing and effluent-minimizing capabilities. This technology aims to decrease process water requirements and hence reduce associated distillation cost, effluent and its treatment cost, which comprises a major portion of overall energy costs, which, in turn, account for about 30% of total production costs [2]; VHG thereby achieves increased productivity through higher ethanol concentra-

Table 1 Merits of advanced VHG fermentation technology compared to conventional low gravity process

Feature	Low or normal gravity	Very high gravity
Feedstock concentration		
Brewing-wort gravity	11–12°P	18°P or more
Fuel ethanol-mash or fermenting medium gravity	16–20 g, max. 24 g/100 ml dissolved solids	30 g or more, max. up to 39 g/100 ml
Final ethanol concentration		
Ethanol content of beer	4–5% (v/v)	14–16% (v/v)
Fuel ethanol	10–12% (v/v)	Increased to >15 or 18% (v/v)
Plant capacity	Fixed	Increased because of more fermentor space created through removal of insoluble matter
Plant efficiency		
Labour costs	Fixed/litre ethanol production	Relatively decreased
Energy costs	Fixed/litre ethanol production	Relatively reduced (about 4% savings) due to less water in fermentor and in still to process; Avoidance of energy loss due to handling insolubles
Potable water savings		
Starch-to-water ratio	1:3	1:2 or 1:1.8, depending on substrate savings up to 58.5%
Effluent	10–15 l/l ethanol	6–9 l/l ethanol recovered
Enzymes' (liquefying & saccharifying) activity	Low activity due to high dilution rate	High activity due to decreased starch-to-water ratio
Enzymes' stability	Less stable	Highly stable due to increased substrate concentration
Spoilage bacteria	Acetic and lactic acid bacteria thrive well, decreases fermentation efficiency	Spoilage bacteria cannot survive better under high osmotic conditions
Co-products/by-products		
Quality	Feed grade by-products	Food quality co-products/distillers grains
Spent yeast	Low protein yeast	High protein spent yeast
Fermentor downtime	Fixed hours	Relatively reduced hours, high-productivity ethanol production

tions in the final broth without major additional investment/infrastructure. In practice, the increased ethanol concentrations at the end of fermentation can be realized by fermenting medium containing sugar in excess of 250 g/l in order to achieve more than 15% (v/v) ethanol compared with 10–12% (v/v), the range that is generally being observed in most distilleries all over the world. The potential benefits of VHG fermentation technology over conventional approaches include a considerable saving of water, more yield of alcohol and reduced labour as well as energy needs, less capital cost and also minimized bacterial contamination [73], as detailed in Table 1.

The concept of high gravity (HG) ethanol fermentation was proposed in the 1980s and successfully applied thereafter, allowing drastic increase of final ethanol concentration from the previous level of 7–8% (v/v) [3]. The concept was further extended to define VHG fermentation technology on the basis of the research progress in yeast physiology that provided new insights into yeast ethanol tolerance. Research has revealed that many strains of *Saccharomyces cerevisiae* can tolerate far higher concentrations of ethanol much better than previously believed, without any conditioning or genetic modifications [13, 78]. In the case of starch as substrate, VHG technology is defined as "the preparation and fermentation to completion of mashes containing 27 g or more dissolved solids per 100 g mash" [76].

However, the yeast cells are subjected to high osmolarity stress at initial stages of fermentation when the sugar level of the medium increases above their normal tolerance limits (>30% w/v). Therefore, sluggish fermentations are often observed, and ultimately the efficiency of ethanol production drops [49]. High osmotic pressure, low water activity

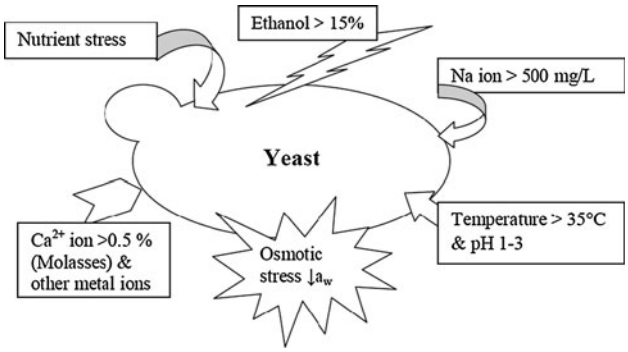

Fig. 1 Prominent stresses experienced by yeast under VHG fermenting conditions

(a_w), and toxic effects of higher ethanol levels are the key factors [32, 69], along with other stress factors, responsible for inhibition of yeast growth and decreased fermentation performance under high substrate concentrations (Fig. 1).

Although certain osmotolerant yeasts such as *Saccharomyces rouxii* can grow well in high-sugar media, their ethanol production levels are relatively low. It has been observed that an increase in osmotic pressure led to an increased intracellular ethanol accumulation [49] in yeast cells that, in turn, exerts a detrimental effect on intracellular enzymes involved in ethanol production. Therefore, yeast-fermenting ability and viability are severely compromised under high osmo-stress conditions. Apart from high initial gravities of the medium, the yeast cells continue to suffer from high concentrations of ethanol at the end of fermentation in a batch mode. In order to reduce or eliminate the negative effects caused by both the increased gravities as well as ethanol levels, research efforts are being carried out to understand the yeast mechanisms for adapting to extreme conditions, focusing majorly on the ethanol excretion and tolerance capacities under such conditions.

VHG brewing

The brewing industry has witnessed significant cost-savings by using this technology. Because during VHG brewing a wort containing 160 g or more of dissolved solids per kilogram is allowed to ferment, followed by maturation, and later diluted with cool carbonated water to a prescribed gravity or alcohol concentration, the brewery capacity can be improved by 50% without needing additional investment [29]. The VHG fermentation is not only an economical process but also aids in production of beer having improved flavour stability and better precipitation of chill haze compared with that of the traditional route, through which about 12°P worts are fermented to produce beers having 5% (v/v) ethanol. Many brewing companies around the world, especially in Canada, are adopting this technology and exploiting its advantages. However, as the wort density increases, the yeast cells are exposed to stress conditions that include nutrients limitation, especially free amino nitrogen (FAN) and dissolved oxygen [14]. Also, concerns such as longer fermentation times, less foam stability, different flavour characteristics and poor hop utilization than in normal gravity fermentations [43] need to be addressed before realizing the full potential of the technology. Moreover, a combination of HG brewing with other modern practices, such as the use of tall cylindroconical fermentors, results in increased hydrostatic pressure, carbon dioxide level, and decreased oxygen level, as well. Many authors have suggested various methods to overcome these drawbacks such as higher fermentation temperature, nutritional supplementation [14, 15, 24, 54], mutant yeast strain [9], more efficient aeration than conventional brewing [15, 18], higher pitching rate [25, 45, 70] and immobilized yeast [46, 47, 51, 66, 83].

Effect of nutrient supplementation

Casey et al. [15] demonstrated the effect of nutrient supplementation in HG brewing by adding a nitrogen source, ergosterol and oleic acid to a 31% dissolved solids wort, through which beers containing ethanol up to 16.2% (v/v) were produced. Dragone et al. [24] successfully demonstrated improvement of ethanol productivity ($0.69\ g\ l^{-1}\ h^{-1}$) of 20°P wort at 15°C, under pilot plant conditions, using a full factorial design to study the influence of different experimental variables; one of these variables included nutrient supplements, namely 0.8% (w/v) yeast extract, 24 mg/l ergosterol and 0.24% (v/v) Tween 80. A recent study indicated that fermentation time can be reduced and at the same time growth of immobilized yeast can be improved by supplementing the wort with Tween 80 and ergosterol at the beginning of fermentation in a fed-batch culture [54]. Ergosterol and Tween 80 (a source of oleic acid, an unsaturated fatty acid) can override the requirement for oxygen in VHG worts, which is essentially required in small amounts by yeasts to maintain the integrity and function of their cell membranes. Moreover, oxygen is relatively poorly soluble in mashes that contain increased amounts of solids. Debourg [18] illustrated that the use of yeast pre-oxygenation prior to pitching and the control of pitching rate are essential prerequisites for consistent and predictable VHG fermentation performances.

Yeast pitching rate

One of the promising methods to improve yeast fermentation performance under VHG conditions is to increase pitching rate. Nguyen and Viet Man [45] showed that higher pitching rate resulted in higher maximum yeast cell number in the culture, higher sugar uptake and ethanol production rates, but

higher diacetyl level in the green beer resulted as well. The results also indicated that this method itself was even more efficient than supplementing wort with nutrients (Tween 80 and yeast extract), or a combination of these two methods, in respect of the sugar uptake and ethanol production rates and ethanol and diacetyl concentrations in the green beer. Diacetyl is a by-product during yeast isoleucine/valine biosynthesis that imparts an undesired butter-like flavour to beer. Erten et al. [25] observed faster fermentation rates, higher yeast counts, higher levels of 2-methyl-1-propanol and lower amounts of 2- and 3-methyl-1-butanols with higher pitching levels. These higher alcohols are undesirable in beer in large amounts. The formation of higher levels of diacetyl and 2,3-pentanedione was observed at lower pitching rates. These results share similarity with a previous report [70]. On the contrary, Verbelen et al. [80] reported that the influence of pitching rate on aroma compound production was rather limited, with the exception of total diacetyl levels, which strongly increased with the pitching rate.

Formation of beer flavour compounds under VHG conditions

A wide range of flavour compounds are synthesized and released by yeast during the brewing process. The important flavour-active compounds in beer include higher alcohols (also called fusel oils), esters and vicinal diketones (VDKs). The formation of increased levels of ethyl acetate and decreased levels of higher alcohols has been reported [22, 63, 70] in worts with higher gravity. Dragone et al. [22] reported the formation of beers with unbalanced flavour profiles under continuous fermentation of HG worts (16.6 and 18.5°P). Increased levels of acetate ester and ethyl hexanoate concentrations were observed under the high density (16 and 18°P) conditions. Also, under these densities, ethyl acetate, isoamyl acetate and phenyl ethanol exceeded their threshold levels [63]. Dragone et al. [23] evaluated the flavour compounds' formation and fermentative parameters of continuous HG brewing with yeasts immobilized on spent grains at different temperatures. In this case, apparent and real degrees of fermentation, rate of extract consumption, ethanol volumetric productivity and consumption of FAN increased upon increasing the temperature from 7 to 15°C. The beer brewed at increased wort osmolarity contained increased levels of diacetyl and pentanedione and lower levels of dimethyl sulfide and acetaldehyde. Esters and higher alcohols displayed small variations irrespective of wort osmolarity or repitching [64].

Foam stability in beer

The interaction between iso-α-acids, which are formed during wort boiling from hop-derived precursors (α-acids), and barley polypeptides is generally known to be responsible for foam stability in beer. Foam stability is reduced during the HG brewing process compared with its low gravity counterparts. Cooper et al. [16] discovered the major causative factor for lack of foam stability during storage of HG beer—proteolytic degradation due to higher levels of yeast proteinase. Proteinase A activity under increased wort gravity is responsible for loss of foam-active hydrophobic polypeptides in wort. Reduced foam stability is, in part, due to poor extraction of high molecular weight foam-active hydrophobic polypeptides from barley during mashing. Proteinase A-induced alterations in the hydrophobicity of hydrophobic polypeptides, such as lipid transfer protein (LPT1), were found to play a key role in instability rather than molecular size of polypeptides [11].

VHG bioethanol fermentation studies

VHG ethanol fermentations have been carried out using media containing simple sugars (glucose, sucrose, etc.) as well as complex carbohydrates (starch, dextrin, etc.). A glucose-containing medium with more than 300 g/l of dissolved solids, along with other nutrients such as free amino nitrogen, yeast extract, sterols, etc., is typically used for VHG fermentation to study ethanol production. Rapid fermentation and high final ethanol concentration are of major importance in the ethanol industry. Process optimization, strict elimination of known losses of substrate or ethanol (a function of plant losses, bacterial and wild yeast conversion of sugars to other end products) as well as sugar bleed, which occurs when yeast stresses increase due to organic acids, ions and other inhibitory end products, have been suggested to achieve more than 90% theoretical ethanol yield [30]. In addition, research has been conducted in yeast physiology to understand the process causing high levels of ethanol-induced inhibition of the fermenting ability and viability of yeast.

Effect of osmoprotectants and nutrients

In order to counter the adverse effects induced by the high density medium such as stuck and sluggish fermentations, many researchers studied the effect/role of osmoprotectants and yeast growth factors by supplementing media with certain nutrients. The stimulation or acceleration of alcoholic fermentation was observed when various adjuncts, assimilatory nitrogen sources, protein–lipid complexes, particulate materials and osmoprotectants were added to the fermentation media. Nutritional adjuncts such as ergosterol, oleic acid and other fatty acids, vegetable oils, skimmed milk powder, chitin, polysaccharides and fungal mycelium supplementation have been shown to benefit the fermenting

yeast [61], and their growth-promoting effects have been characterized. Assimilable nitrogen is necessary for yeast growth and multiplication; it influences the ethanol tolerance of yeasts and the rate of ethanol production. The addition of FAN sources such as urea, yeast extract and ammonium salts [67, 78] leads to higher final ethanol concentrations in the VHG fermented media. The final ethanol concentration achieved was increased by 17% (to 103 g/l) when excess assimilable nitrogen was added to the batch VHG ethanolic fermentations by *Saccharomyces cerevisiae*. The supplementation of the media with 12 g yeast extract, 0.3 g cell walls, 3 g glycine and 20 g soya flour per litre halved the fermentation time to 28 h, thereby enhancing the ethanol productivity by more than 50% [1]. Pereira et al. [52] developed a medium based on corn steep liquor (CSL) and urea for VHG ethanol fermentations, which resulted in significantly enhanced final ethanol productivity and yeast viability (up to 330 g/l glucose) with a corresponding productivity of 2.4 g l^{-1} h^{-1}. It was reported recently that urea supplementation during VHG fermentation not only balances the nitrogen deficiency, but also assists in prolonging the logarithmic phase of yeast cells in order to achieve high ethanol productivity [72].

Effect of osmoprotectants including proline, glycine betaine, glycine, tryptone, adenine-uracil-cytosine, ammonium, calcium and magnesium salts have been tested under HG conditions [74]. Ethanol productivity can be enhanced by supplementing the rate-limiting reaction substrate in yeast ethanol biosynthesis namely acetaldehyde. The presence of soya flour and yeast cell wall preparations enhances the fermentation rate, the amount of sugar consumed and the final ethanol concentration in the fermentation media. Breisha [10] reported that addition of nitrogen as ammonium sulfate at a rate of 5 mg per gram of consumed sucrose produced 11.55% ethanol with complete consumption of 25% sucrose after 48 h of fermentation. Addition of yeast extract at a level of 6 g/l together with thiamine at a level of 0.2 g/l led to complete utilization of 30% sucrose with resultant 14% ethanol production. However, the selected yeast strain was not able to ferment 35% sucrose under the same optimum conditions. Addition of air at a rate of 150 dm^3 min^{-1} m^{-3} of reactor volume during the first 12 h of fermentation led to complete consumption of 35% sucrose and 16% ethanol was produced.

Studies using saccharide substrates

Several studies have shown that the ethanol levels are improved by addition of nutrients to a simple medium containing 300 g/l of glucose: for example, addition of soy flour 4% (w/v) increased the ethanol levels to 12.8% (w/v) [82]; addition of horse gram flour (*Dolichos biflorus*) and finger millet flour (*Eleusine coracana* L.) [61, 62] and malted cowpea (*Vigna unguiculata* L.) flour [57] increased ethanol concentrations to more than 15% (v/v) within 72 h fermentation time with productivities greater than 2 g l^{-1} h^{-1}. Low-cost nutrient sources such as CSL, urea and magnesium sulfate have been applied to afford maximum ethanol production [18.6% (v/v)] in batch VHG fermentations of up to 330 g/l glucose [52]. Fermentation of mixtures of sweet stem sorghum juice and sorghum grain (malted) under VHG conditions yielded 16.8% (v/v) [12]. Recently, ethanol production from sweet sorghum juice under VHG fermentation was investigated using various carbon adjuncts and nitrogen sources [36]. In this study, the maximum ethanol efficiency reported was above 15.0% (v/v), using sucrose adjunct; using sugarcane molasses as adjunct, a slight reduction to approximately 14.0% (v/v) was observed. Batch and repeated-batch ethanolic fermentation of sweet sorghum juice were carried out by the same research group in scale-up studies using 500-ml, 5.0-l and 50.0-l fermentors yielded ethanol concentrations 120.24 $\pm$ 1.35, 139.51 $\pm$ 0.11 and 119.53 $\pm$ 0.2 g/l, respectively [48]. An ethanol yield of 14.8% (v/v) has been reported [35] from fermentation of blackstrap sugarcane molasses as sole substrate under VHG conditions (47.7 g/100 ml). In that report, the addition of 20 mM diammonium phosphate improved the ethanol yield. From the same report, VHG fermentation of sugarcane juice fortified with molasses (34–35% w/v) yielded ethanol as high as 15.8% (v/v) at 30°C within 48 h. Recently, Pradeep and Reddy [58] reported the role of nutrients in molasses fermentation under VHG conditions; a final ethanol concentration of 13.6% (v/v) was observed within 48 h by fermentation of nutrient-supplemented molasses possessing 34°Bx dissolved solids.

Studies using starchy substrates

Significant contributions have been made by several researchers for strengthening VHG technology using starches. Even though marked improvements in the ethanol yields have been reported using wheat mashes, most studies are now exploring the possibility of using other starch substrates. For the preparation of VHG mashes, certain strategies must be followed including grain-to-water ratio enhancement, the use of mash viscosity-reducing enzymes and removal of insoluble solids [31]. Remarkable improvement in the ethanol yield to over 23% (v/v) has been reported [76] from VHG fermentation of wheat mashes containing 38–39% (w/v) dissolved solids. A list of starch substrates that have been tested and shown to be suitable for VHG fermentations is given in Table 2. One recent report included the successful VHG fermentation of sweet potato mash [88], which was pretreated using xylanase to reduce viscosity. The feasibility and potentiality of HG raw

Table 2 VHG ethanol fermentation studies using different starch substrates

Substrate	Conc. of dissolved solids	Maximum ethanol produced	Fermentation time (h)	Reference
Wheat mash	35% (w/v)	17.1% (v/v)	72	Thomas and Ingledew [77]
Wheat mash	37.9% (w/v)	23.8% (v/v)	130	Thomas et al. [76]
Hull-less barley	32% (w/v)	17.1% (v/v)	96	Thomas et al. [73]
Oats (hull-less)	>30% (w/v)	353.2 ± 3.7 l/t (dry wt.)	72	Thomas and Ingledew [79]
Rye	32–34% (w/v)	434.5 ± 5.1 l/t (dry wt.)	48	Ingledew et al. [31]
Rye and triticale	>30% (w/v)	15.7–16.1% (v/v)	96–120	Wang et al. [85]
Sweet & grain sorghum	34% (w/v)	16.8% (v/v)	96	Bvochora et al. [12]
Corn mash	35% (w/w)	126–130 (g/kg)	72	Devantier et al. [19]
Malto-dextrin	>30% (w/v)	129 (g/l)	72	Devantier et al. [20]
Pearl millet	35% (w/v)	16.8% (v/v)	72	Wu et al. [86]
Corn mash	>30% (w/v)	17% (v/v)	48	Wang et al. [84]
Barley (dehulled bold)	30% (w/w)	14.3% (v/v)	72	Gibreel et al. [27]
Potato mash	>30% (w/v)	16.61% (v/v)	72	Srichuwong et al. [67]
Finger millet mash	>30% (w/v)	15.6% (v/v)	72	Pradeep et al. [55]
Cassava starch	40% (w/v)	15.03% (v/v)	72	Yingling et al. [87]
Sweet potato mash	>30% (w/v)	~17.0% (v/v)	36	Zhang et al. [88]

sweet potato for bioethanol production by the simultaneous saccharification and fermentation (SSF) process was explored by the same team at laboratory, pilot and industrial scales, resulting in maximum ethanol concentrations of 128.51, 109.06 and 97.94 g/l, respectively [89].

Mash viscosity

The problem of high mash viscosity has been encountered during VHG mash preparation using some starch substrates. High viscosity can cause handling difficulties during processes, resistance to solid–liquid separation, incomplete hydrolysis of starch to fermentable sugars and, therefore, could result in low fermentation efficiency [68, 88]. To avoid such viscosity build-up, enzymatic pretreatment (before mashing) with mixed enzyme preparations is recommended to produce mashes with sufficiently high dissolved solids content, and having an acceptable viscosity. These enzymes help increase the water-to-grain ratio by freeing water bound by mash components. Cell-wall degrading enzymes such as cellulase, hemicellulase, xylanase and arabinase have been employed for mash viscosity reduction. Additionally, the use of multiple pectin degrading enzymes greatly contributed to viscosity reduction in using substrates like potato starch [67]. Jones and Ingledew [34] assessed the ability of proteases to hydrolyse wheat proteins to FAN and to reduce the viscosity of VHG wheat mashes. Biocellulase or β-glucanase was applied to hydrolyse β-glucan of barley and oat mashes. A successful viscosity reduction at the rate 8.2 BU/s, from 2,460 to 420 BU, was reported on treating barley mash (prepared with a water-to-grain ratio of 3:1) with β-glucanase (0.02%, w/w) [73].

Process optimization studies

The ultimate goal of the VHG ethanol fermentation process is to achieve higher final ethanol concentrations within the shortest period possible. The improved ethanol concentrations in the medium could be realized by process optimization. In particular, concentrations of nutrients should be optimized because nutrients supplementation has been proved to play an important role under VHG conditions. Using uniform design and a nonlinear stepwise regression analysis, Wang et al. [84] calculated optimal concentrations of nutrients and other factors required for VHG fermentation of corn mash. By using a surface response design and multiple regression analysis, Zhang et al. [88] optimized the factors influencing viscosity reduction in the fermentation of sweet potato mash to ethanol under VHG conditions. Statistical experimental designs such as Plackett–Burman design for selection of critical nutrients and response surface methodology, which was based on a three-level four-factor Box–Behnken design for optimization of medium composition, were successfully employed for designing a low-cost VHG medium based on glucose [52]. Recently, correlations between reduction–oxidation potential profiles, which were controlled at certain levels by manipulating aeration, and the growth patterns of *Saccharomyces cerevisiae* during VHG fermentation were studied [38]. The results indicate that redox potential profiles are useful to pick up subtle differences as yeasts transition from one phase to another; thereby proper substrate feeding and/or product recovery strategies could be developed to maximize ethanol productivity and to process automation. The thermodynamic approach, calorimetric measurement, can be used to monitor yeast growth

patterns and evaluate fermentation performance [72]; an online calorimetric VHG fermentation process could be developed by integrating the data collected on heat evolution. Using the transcript analysis with aid of affinity capture (TRAC) method, Rautio et al. [60] obtained a dynamic picture of the physiological state of fermenting yeast by analysing selective genes at frequent intervals; thereby monitoring and optimization of yeast performance could be possible in complex process environments.

A favourable physiological environment for the yeast cells, which is generally lacking under VHG conditions because of a variety of stresses, to obtain a high yield of ethanol can be created by improvements in the process engineering design and operation. For example, increasing the number of tanks-in-series systems for new plants or adding baffles inside the existing tanks can significantly decrease the overall backmixing and alleviate the ethanol inhibition effect. This approach is believed to have more economic feasibility and is a focus of continuing development [3]. More studies are needed to support the approach as only scanty information is available, specifically work by Bayrock and Ingledew [7] and Lin et al. [37], who reported the advantage of continuous VHG ethanol fermentation in a multistage fermentation system. Also, Bai et al. [4] investigated a bioreactor system composed of a continuously stirred tank reactor (CSTR) and three tubular bioreactors in series.

Mutants and gene level studies

Mizuno et al. [41] successfully demonstrated HG brewing using strain 2DGR19, a 2-deoxyglucose-resistant mutant of brewers' yeast *Saccharomyces cerevisiae* NCYC1245; the mutant performance was also demonstrated on a pilot scale for obtaining beers with low acetic acid and higher ethanol productivities compared with the parent strain. To monitor the physiological condition of yeast in the brewing process, especially under VHG conditions, Rautio et al. [60] used the TRAC method to monitor cells' adaptation to changing surroundings by transcriptional regulation. Stress responsive genes namely *HSP104* and *TPS1* (trehalose-6-phosphate synthase) which have stress responsive elements (STRE) in their promoters have been shown to be induced during HG wort fermentation and they exhibit a similar expression pattern. Blieck et al. [9] successfully demonstrated the microarray-based gene analysis strategy to isolate better-performing yeast variants under VHG wort (>22°P) conditions. A good correlation was demonstrated between flavour compound synthesis and the expression level of specific genes involved in the biosynthesis of aroma compounds under HG brewing conditions [63]. Global transcription machinery engineering (gTME) technology, which aims to modify the transcription factor behaviour to reprogram a series of gene transcriptions, was applied to an industrial brewer's yeast for improving stress tolerance [26]. In that study, site-specific mutagenesis of the *SPT15* gene in wild-type yeast generated a mutant, *SPT15-300*, which exhibited increased glucose uptake rate and higher ethanol productivity than the wild-type strain under HG conditions. Guimaraes and Londesborough [28] estimated the intracellular and extracellular ATP, ADP and AMP (i.e. 5′-AMP) levels during fermentations of high (15°P) and VHG (25°P) worts. Substantial amounts of extracellular AMP were found with increase in gravity, ATP was the dominant intracellular adenine nucleotide and the adenylate energy charge (EC) remained high (>0.8) until residual sugar concentrations were low and specific rates of ethanol production were less than 5% of the maximum values in early fermentation. James et al. [33] characterized the genomes of a number of stress-tolerant mutants, which are tolerant to high temperatures and to growth in high specific gravity wort. They found that the mutant strains had undergone extensive gross chromosomal rearrangements compared with the parent strain such that they tolerated the stress well; it was also found that the lager yeast genome can exhibit a high degree of plasticity under high wort gravity and high temperature conditions.

Immobilized yeast applications

Immobilization may lower the stress of yeast under VHG fermentation depending on the carrier used for immobilization. Szajáni et al. [71] and Smogrovicova et al. [66] observed that fermentations of yeasts immobilized on cellulose were very similar to free yeasts under VHG conditions. On the other hand, Smogrovicova et al. [66] observed a significant increase of fermentation rate of VHG wort for brewery yeast entrapped in calcium pectate or alginate. They confirmed that immobilization protects the microbial cells against the possible toxic effect of substrates or products whereby entrapment in gel appeared to be more favourable than adsorption. If the gel matrix contained calcium cations, the ethanol tolerance was even more improved. These results are in agreement with those obtained by Norton and D'Amore [46], Norton et al. [47] and Patkova et al. [51]. Patkova found that by using calcium alginate-entrapped yeast, 24% (w/w) wort was successfully fermented within 8 days. This was half the time needed for fermentation by free yeast. Virkajarvi et al. [83] used porous glass beads as the carrier for wort fermentation, and found that the optimal wort gravity in regard to ethanol productivity was between 18 and 21°P. The highest ethanol concentration in the beers was over 10% (v/v). The aroma compound profiles of the beers produced were quite similar, except for acetaldehyde.

The research related to progress of both ethanol and beer fermentation using the immobilized yeast cells was reviewed by Najafpour et al. [44], Verbelen et al. [81] and Silva et al. [65]. Recently, immobilized yeast cells were used, inside Ca-alginate and κ-carrageenan polymers, in up to four successive batches to ferment a simple HG medium for bioethanol production [56]. A combination of continuous fermentation and yeast immobilization is performed very often, because immobilized biomass removes the washout limitation of continuous operation with free cells, and results in a higher productivity.

Effect of temperature and aeration

Using molasses VHG medium, Jones et al. [35] reported that the rate of sugar utilization by yeast is slow at 15°C; only 44% of the dissolved solids are utilized even in 240 h fermentation time. Whereas at 35°C the fermentation ceased within 72 h, and maximum sugar utilization (>75% dissolved solids) is observed at 20 and 25°C. An optimal temperature of 27–30°C has been recommended for ethanolic fermentation of VHG wheat mashes (36.5 g/100 ml dissolved solids) to obtain more than 20% (v/v) ethanol within 55 h in the presence of 16 mM urea [34]. In contrast, an inverse interactive effect between temperature and urea was observed; however, it was yeast-dependent [72]. To propagate healthy yeast population, proper aeration strategy should be implemented as small amounts of oxygen are essential at certain fermenting stages of yeasts for maintaining the cell membrane integrity through synthesizing sterols and unsaturated fatty acids. Yeast growth and ethanol productivity could be effectively augmented by providing an optimal aeration duration and interval. In addition, the better management of glycerol by-product production in biofuel ethanol fermentation could be achieved. Aeration at the rate 0.82 l/min between early log and mid log phases of growth (i.e. 8–18 h) was found to be more effective in enhancing the yeast viability and ethanol productivity in a VHG ethanol fermentation study [39].

Reserve carbohydrates profile in yeast

Trehalose and glycogen are the main reserve carbohydrates in yeast. Strains that possess the ability to ferment media with high sugar concentration (i.e. osmotolerant) accumulate high trehalose concentrations [17]. However, trehalose is also synthesised in response to stress and may act as a general stress protectant for yeasts. Glycogen is the sole source of metabolic energy for lipid synthesis and hexose transport during initial hours of fermentation [59]. Studies on yeast trehalose and glycogen metabolism under VHG conditions have been discussed [40, 50]. Increased levels of trehalose accumulate inside when yeast cells are exposed to osmotic stress, high ethanol concentration, and heat and cold shocks. A significant reduction in intracellular trehalose accumulation has been observed in fermentation of HG molasses supplemented with additives including soybean meal, groundnut meal or castor oil meal [5]. The maximum intracellular trehalose content in calcium alginate-entrapped yeast was three times lower compared to free yeast in a VHG wort fermentation. This proved that yeast immobilization in gel matrix protects the cells against osmotic stress and toxic effects of the produced ethanol [51].

Osmo-induced glycerol synthesis

Yeast cells' exposure to increased external osmolarities leads to cell dehydration and collapse of plasma membrane ion-gradients; thereby a loss in viability could generally be expected in fermentations in media of increased density. In response to osmolarity change, to optimize survival and proliferation, cellular glycerol concentration in yeast cells is increased through the activation of the high osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway. Under hyperosmolar glucose concentrations, multiple MAPK cascades are initiated by activation of 6-phosphofructo-2-kinase (PFK2). The upper part of glycolysis is activated through activated PFK2; thereby glycerol accumulates three times more than that using yeast lacking it [21]. The up-regulation of Gpd 1p (NAD-dependent glycerol-3-phosphate dehydrogenase), a key enzyme of glycerol synthesis, is essential for glycerol synthesis and growth under osmotic stress [53]. The quantities of accumulated or total synthesized glycerol have been shown to be reduced in VHG fermentations supplemented with nutrients, in the presence of osmoprotectants [61, 62] and using immobilized yeast cells [56].

Economics/cost-savings of VHG fermentations

In VHG fermentation, nearly 40% reduction in the process water usage can be achieved through the high solids mash preparation (a decrease in water-to-starch ratio), thereby remarkable energy savings from further processing steps could be anticipated because there is less fluid to heat, cool and distill. As much as 58.5% water-saving potential has been calculated for wort fermentation by increasing the gravity from 16 to 31°P [75]. The calculations showed that 78,232 kg water can be saved by preparing 31°P wort instead of 16°P for 44,558 kg wheat. Water requirement dramatically reduced from 133,674 kg (water-to-grain ratio

3:1 under normal gravity) to 55,442 kg (58.5% reduction under VHG conditions).

Among energy utilization costs, 80% comes from downstream processing. The distillation cost constitutes almost 30% of the total operating cost. Owing to a relatively low level of water consumption, distillation and stillage evaporation costs can be significantly reduced in the VHG process. A considerable saving in energy, about 4%, has been reported when final ethanol concentrations increased from 12 to 18% under HG fermentation conditions [8]. Furthermore, risk of bacterial contamination could be minimized due to the fact that bacteria cannot thrive well under increased osmotic conditions; therefore, costs associated with antibiotics use are lowered.

Also, in recent years, highly efficient α-amylases, glucoamylases and proteases have been available at low cost. As the wet-milling process is gaining in importance due to emerging value of by-products, the use of insoluble solids-free and starch-rich VHG medium can be anticipated at industry level [3].

Addition/modification of inputs/equipment required in existing process

Certain additional practices are recommended to increase the fermentation efficiency under VHG conditions, depending on the substrate used. For sugary substrates' fermentation, especially using cane or beet molasses, gravity change through decreased dilution with water and addition of extra or increased amounts of nutrients/osmoprotectants will serve the purpose. Additionally, pretreatment/preclarification of medium before fermentation by ceramic microfiltration/activated carbon filter could effectively remove impurities such as sulfated ash, inorganic salts and fermentation inhibitory compounds. On the other hand, to process grain-based starches under increased gravities, additional practices/inputs required are substrate- as well as process-dependent as exemplified in the case of the standard dry-grind ethanol process (Fig. 2).

Grain pearling

Polishing or pearling of grain has been recommended to increase the starch content of mashes before slurry preparation. The outer layers of grain, containing mainly bran and aleurone portions, can be removed progressively by successive abrasions. An increase of 7–8% starch has been reported in pearled rye (from 62 to 69%) and in pearled triticale (from 63 to 71%) [85]. Corn and other grain polishing/decorticating machines are extensively available on the market.

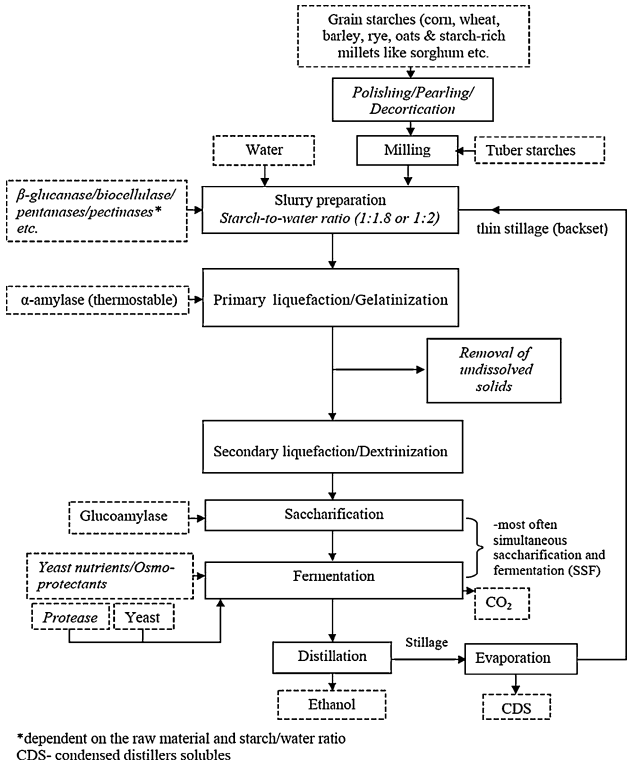

Fig. 2 Process flow diagram of the VHG ethanol production from starches by dry-grinding process. Some of the additional practices/inputs (indicated in *italics*) required for higher ethanol titres and enhanced process efficiency

Pre-mashing

In order to reduce slurry viscosity, a pre-mashing procedure before starch liquefaction has been recommended in the case of barley and oat mashes preparation. Incubation of slurry with microbial lignocellulolytic enzymes potentially reduces mash viscosity. Temperature- and pH-controlled stirred tanks could be ideal at this step for maximum viscosity reduction.

HG mashes preparation

Thermostable amylases are used in general for high starch gelatinization, and a dextrinization step for complete liquefaction. In processes where VHG mash preparation from one type of grain is found difficult simply by adjusting the water-to-grain ratio, double-mashing is recommended [75]. Twin screw extruders have successfully been used for gelatinize slurry having substrate concentrations as high as 50–70% (w/w); they can more efficiently handle (gelatinize) concentrated starch slurries than stirred batch vessels [6]. Gelatinization temperature can be decreased under application of shear in the extruder, thereby lowering water and energy requirements. The single decoction plus infusion

method is better for obtaining high fermentable sugars than double decoction mashing in the brewing process [42].

Removal of insolubles

Insoluble solids need to be removed prior to saccharification of liquefied starch. Decanter centrifuges are ideally suited for removal of insolubles that could otherwise create obstacles in further process steps as well as in downstream processing.

Fermentor design aspects

As a result of the high viscosity of VHG mashes, heat transfer to maintain the optimal temperature for fermentation may be a bit difficult, especially in tropical regions. The heat build-up could be overcome by use of helical baffles heat exchanger/scraped surface heat exchangers (SSHE), which are especially suitable for high viscosity fluids. To transfer high viscous mash between units, increased slurry temperatures (80–90°C) after jet cooking in the case of starches is sufficient to reduce viscosity to an extent easily handled by centrifugal pumps. For other substrates such as molasses, hydraulic gear pumps could serve the purpose. Feasibility of coupling multistage continuous culture fermentation (MCCF) technology to VHG fermentation has been tested [7]; the merging yields a 42% increase in ethanol production. At present, no studies focused on fermentor design aspects for VHG fermentations and, therefore, studies are needed in this area.

Concluding remarks

VHG fermentation technology has great potential in both ethanol and beer production; however, the process could be effective only after eliminating the negative consequences of yeast stress caused by increasing osmotic pressure and toxicity of the produced ethanol. The yeast stress can be lowered by nutrient supplements such as free amino nitrogen, ergosterol and other compounds which yeasts synthesize in response to stress; others include more efficient aeration, higher pitching rate, using special osmo- and ethanol-tolerant mutant yeast, cell immobilization, especially entrapment of cells in gel matrix, and process optimization.

For optimum and efficient industrial production of ethanol over the range of normal gravity to VHG, fermentation temperature should not exceed 30°C. In recent years, the availability of environmentally friendly, efficient process enzymes for starch conversion in a cost-effective way guarantees the steady supply of HG mash to industry. The yeast cells' immobilization approach could also be used in the bioethanol industry for successful elimination of substrate and product stresses, and to maintain maximum yeast viability for complete fermentation. On the other hand, process optimization can be more useful for effective and more complete utilization of substrates and costly nutrients including osmotolerants.

Whereas in ethanol production the productivity of the process is the most important factor, in beer production the goal is typically to maintain desirable flavour characteristics of the produced beer, which can be influenced by any change in the process. Above all, any improved and effective process development pertaining to VHG technology should be technically and economically viable, and acceptable to industry.

Acknowledgments The present work was supported by the University Grants Commission (UGC), New Delhi, through Major Research Project (MRP) grant, and the Scientific Grant Agency of the Ministry of Education of the Slovak Republic VEGA 1/0096/11.

References

1. Bafrncová P, Šmogrovičová D, Sláviková I, Pátková J, Dömény Z (1999) Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*. Biotechnol Lett 21:337–341
2. Bai FW (2007) Process oscillations in continuous ethanol fermentation with *Saccharomyces cerevisiae*. PhD thesis, Chemical Engineering, University of Waterloo, Canada
3. Bai FW, Anderson WA, Moo-Young M (2008) Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv 26:89–105
4. Bai FW, Chen LJ, Anderson WA, Moo-Young M (2004) Parameter oscillations in very high gravity medium continuous ethanol fermentation and their attenuation on multi-stage packed column bioreactor system. Biotechnol Bioeng 88:558–566
5. Bajaj BK, Taank V, Thakur RL (2005) Potential industrial applications of yeast capable of fermenting high gravity cane molasses despite physiological stress. Indian J Biotechnol 4:149–152
6. Baks T (2007) Process development for gelatinisation and enzymatic hydrolysis of starch at high concentrations. Thesis, Wageningen University, the Netherlands
7. Bayrock DP, Ingledew WM (2001) Application of multi-stage continuous fermentation for production of fuel alcohol by very-high gravity fermentation technology. J Ind Microbiol Biotechnol 27:87–93
8. Belcher A (2005) The world looks to higher-tech to advance fuel ethanol production into the 21st century. Int Sugar J 107(1275): 196–199
9. Blieck L, Toye G, Dumortier F, Verstrepen KJ, Delvaux FR, Thevelein JM, Van Dijck P (2007) Isolation and characterization of brewer's yeast variants with improved fermentation performance under high gravity conditions. Appl Environ Microbiol 73:815–824
10. Breisha GZ (2010) Production of 16% ethanol from 35% sucrose. Biomass Bioenerg 34:1243–1249
11. Brey SE, De Costa S, Rogers PJ, Bryce JH, Morris PC, Mitchell WJ, Stewart GG (2003) The effects of proteinase A on foam-active polypeptides during high and low gravity fermentation. J Inst Brew 109:194–202
12. Bvochora JM, Read JS, Zvauya R (2000) Application of very high gravity technology to the cofermentation of sweet stem sorghum juice and sorghum grain. Ind Crop Prod 11:11–17

13. Casey GP, Ingledew WM (1986) Ethanol tolerance in yeasts. CRC Crit Rev Biotechnol 13:219–280
14. Casey GP, Magnus CA, Ingledew WM (1983) High gravity brewing: nutrient enhanced production of high concentrations of ethanol by brewing yeast. Biotechnol Lett 5:429–434
15. Casey GP, Magnus CA, Ingledew WM (1984) High-gravity brewing: effects of nutrition on yeast composition, fermentative ability, and alcohol production. Appl Environ Microbiol 48:639–646
16. Cooper DJ, Stewart GG, Bryce JH (2000) Yeast proteolytic activity during high and low gravity wort fermentations and its effect on head retention. J Inst Brew 106:197–201
17. D'Amore T, Crumplen R, Stewart GG (1991) The involvement of trehalose in yeast stress tolerance. J Ind Microbiol 7:191–196
18. Debourg A (2010) Yeast management and high gravity fermentation. Cerevisia 35:16–22
19. Devantier R, Pedersen S, Olsson L (2005) Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharification and yeast strain. Appl Microbiol Biotechnol 68:622–629
20. Devantier R, Scheithauer B, Villas-Boas SG, Pedersen S, Olsson L (2005) Metabolite profiling for analysis of yeast stress response during very high gravity ethanol fermentations. Biotechnol Bioeng 90:703–714
21. Dihazi H, Kessler R, Eschrich K (2004) High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress. J Biol Chem 279:23961–23968
22. Dragone G, Mussatto SI, Almeida e Silva JB (2007) High gravity brewing by continuous process using immobilised yeast: effect of wort original gravity on fermentation performance. J Inst Brew 113:391–398
23. Dragone G, Mussatto SI, Almeida e Silva JB (2008) Influence of temperature on continuous high gravity brewing with yeasts immobilized on spent grains. Eur Food Res Technol 228:257–264
24. Dragone G, Silva DP, De Almeida E Silva JB, De Almeida Lima U (2003) Improvement of the ethanol productivity in a high gravity brewing at pilot plant scale. Biotechnol Lett 25:1171–1174
25. Erten H, Tanguler H, Cakiroz H (2007) The effect of pitching rate on fermentation and flavor compounds in high gravity brewing. J Inst Brew 113:75–79
26. Gao C, Wang Z, Liang Q, Qi Q (2010) Global transcription engineering of brewer's yeast enhances the fermentation performance under high-gravity conditions. Appl Microbiol Biotechnol 87:1821–1827
27. Gibreel A, Sandercock JR, Lan J, Goonewardene LA, Zijlstra RT, Curtis JM, Bressler DC (2009) Fermentation of barley by using *Saccharomyces cerevisiae*: examination of barley as a feedstock for bioethanol production and value-added products. Appl Environ Microbiol 75:1363–1372
28. Guimaraes PMR, Londesborough J (2008) The adenylate energy charge and specific fermentation rate of brewer's yeasts fermenting high and very high-gravity worts. Yeast 25:47–58
29. Hackstaff BW (1978) Various aspects of high gravity brewing. Master Brew Assoc Am Tech Q 15:1–7
30. Ingledew WM (2005) Improvements in alcohol technology through advancements in fermentation technology. Getreidetechnologie 59:308–311
31. Ingledew WM, Thomas KC, Hynes SH, McLeod JG (1999) Viscosity concerns with rye mashes used for ethanol production. Cereal Chem 76:459–464
32. Jakobsen M, Piper JU (1989) Performance and osmotolerance of different strains of lager yeast in high gravity fermentation. MBAA Tech Quart 26:56–61
33. James TC, Usher J, Campbell S, Bond U (2008) Lager yeasts possess dynamic genomes that undergo rearrangements and gene amplification in response to stress. Curr Genet 53:139–152
34. Jones AM, Ingledew WM (1994) Fuel alcohol production: optimization of temperature for efficient very-high-gravity fermentation. Appl Environ Microbiol 60:1048–1051
35. Jones AM, Thomas KC, Ingledew WM (1994) Ethanolic fermentation of blackstrap molasses and sugarcane juice using very high gravity technology. J Agric Food Chem 42:1242–1246
36. Laopaiboon L, Nuanpeng S, Srinophakun P, Klanrit P, Laopaiboon P (2009) Ethanol production from sweet sorghum juice using very high gravity technology: effects of carbon and nitrogen supplementations. Bioresour Technol 18:4176–4182
37. Lin YH, Bayrock DP, Ingledew WM (2002) Evaluation of *Saccharomyces cerevisiae* grown in a multi-stage chemostat environment under increasing levels of glucose. Biotechnol Lett 24:449–453
38. Lin YH, Chien WS, Duan KJ (2010) Correlations between reduction–oxidation potential profiles and growth patterns of *Saccharomyces cerevisiae* during very-high-gravity fermentation. Process Biochem 45:765–770
39. Lin YH, Chien WS, Duan KJ, Chang PR (2011) Effect of aeration timing and interval during very-high-gravity ethanol fermentation. Process Biochem. doi:10.1016/j.procbio.2011.01.003
40. Majara M, O'Connor-Cox ESC, Axcell BC (1996) Trehalose: an osmoprotectant and stress indicator compound in high and very high gravity brewing. J Am Soc Brew Chem 54:149–154
41. Mizuno A, Tabei H, Iwahuti M (2006) Characterization of low-acetic-acid-producing yeast isolated from 2-deoxyglucose-resistant mutants and its application to high-gravity brewing. J Biosci Bioeng 101:31–37
42. Montanari L, Floridi S, Marconi O, Tironzelli M, Fantozzi P (2005) Effect of mashing procedures on brewing. Eur Food Res Technol 221:175–179
43. Munroe JH (1995) Fermentation. In: Hardwick WA (ed) Handbook of brewing, 1st edn. Marcel Dekkar, New York, pp 323–353
44. Najafpour G, Younesi H, Ismail KSK (2004) Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. Bioresour Technol 92:251–260
45. Nguyen TH, Viet Man LV (2009) Using high pitching rate for improvement of yeast fermentation performance in high gravity brewing. Int Food Res J 16:547–554
46. Norton S, D'Amore T (1994) Physiological effects of yeast cell immobilization: applications for brewing. Enzyme Microb Technol 16:365–375
47. Norton S, Watson K, D'Amore T (1995) Ethanol tolerance of immobilized brewers' yeast cells. Appl Microbiol Biotechnol 43: 18–24
48. Nuanpeng S, Laopaiboon L, Srinophakun P, Klanrit P, Jaisil P, Laopaiboon P (2011) Ethanol production from sweet sorghum juice under very high gravity conditions: batch, repeated-batch and scale up fermentation. Elect J Biotechnol 14(1). doi:10.2225/vol14-issue1-fulltext-2
49. Panchal CJ, Stewart GG (1980) The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast strain. J Inst Brew 86:207–210
50. Patkova J, Smogrovicova D, Bafrncova P, Domeny Z (2000) Changes in the yeast metabolism at very high gravity wort fermentation. Folia Microbiol 45:335–338
51. Patkova J, Smogrovicova D, Domeny Z, Bafrncova P (2000) Very high gravity wort fermentation by immobilised yeast. Biotechnol Lett 22:1173–1177
52. Pereira FB, Guimarães PMR, Teixeira JA, Domingues L (2010) Optimization of low-cost medium for very high gravity ethanol fermentations by *Saccharomyces cerevisiae* using statistical experimental designs. Bioresour Technol 101:7856–7863

53. Pham TK, Chong PK, Gan CS, Wright PC (2006) Proteomic analysis of *Saccharomyces cerevisiae* under high gravity fermentation conditions. J Proteome Res 5:3411–3419
54. Pham TNL, Doan NHD, Le VVM (2010) Using fed-batch fermentation in very high gravity brewing: effects of Tween 80 and ergosterol supplementation on fermentation performance of immobilized yeast in calcium alginate gel. Int Food Res J 17:995–1002
55. Pradeep P, Goud GK, Reddy OVS (2010) Optimization of very high gravity (VHG) finger millet (*ragi*) medium for ethanolic fermentation by yeast. Chiang Mai J Sci 37:116–123
56. Puligundla P, Poludasu RM, Rai JK, Obulam VSR (2011) Repeated batch ethanolic fermentation of very high gravity medium by immobilized *Saccharomyces cerevisiae*. Ann Microbiol. doi:10.1007/s13213-011-0207-8
57. Pradeep P, Reddy OVS (2008) Effect of supplementation of malted cowpea (*Vigna unguiculata* L.) flour in the enhancement of yeast cell viability and ethanol production in VHG fermentation. Asian J Microbiol Biotech Env Sc 10:767–772
58. Pradeep P, Reddy OVS (2010) High gravity fermentation of sugarcane molasses to produce ethanol: Effect of nutrients. Indian J Microbiol 50:82–87
59. Quain DE, Tubb RS (1982) The importance of glycogen in brewing yeasts. Master Brew Assoc Am Tech Quart 19:29–33
60. Rautio JJ, Huuskonen A, Vuokko H, Vidgren V, Londesborough J (2007) Monitoring yeast physiology during very high gravity wort fermentations by frequent analysis of gene expression. Yeast 24:741–760
61. Reddy LVA, Reddy OVS (2005) Improvement of ethanol production in very high gravity (VHG) fermentation by horse gram (*Dolichos biflorus*) flour supplementation. Lett Appl Microbiol 41:440–444
62. Reddy LVA, Reddy OVS (2006) Rapid and enhanced production of ethanol in very high gravity (VHG) sugar fermentation by *Saccharomyces cerevisiae*: role of finger millet (*Eleusine coracana* L.) flour. Process Biochem 41:726–729
63. Saerens SMG, Verbelen PJ, Vanbeneden N, Thevelein JM, Delvaux FR (2008) Monitoring the influence of high-gravity brewing and fermentation temperature on flavor formation by analysis of gene expression levels in brewing yeast. Appl Microbiol Biotechnol 80:1039–1051
64. Sigler K, Matoulková D, Dienstbier M, Gabriel P (2009) Net effect of wort osmotic pressure on fermentation course, yeast vitality, beer flavor, and haze. Appl Microbiol Biotechnol 82:1027–1035
65. Silva DP, Branyik T, Dragone G, Vicente AA, Teixeira JA, Almeida e Silva JB (2008) High gravity batch and continuous processes for beer production: evaluation of fermentation performance and beer quality. Chem Pap 62:34–41
66. Smogrovicova D, Patkova J, Domeny Z, Navratil M (2000) Improvement in beer fermentation under very high gravity conditions by entrapped yeast. Minerva Biotecnol 12:331–336
67. Srichuwong S, Fujiwara M, Wang X, Seyama T, Shiroma R, Arakane M, Mukojima N, Tokuyasu K (2009) Simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol. Biomass Bioenerg 33:890–898
68. Srikanta S, Jaleel SA, Ghildyal NP, Lonsane BK (2006) Techno-economic feasibility of ethanol production from fresh cassava tubers in comparison to dry cassava chips. Food/Nahrung 36: 253–258
69. Stewart GG, D'Amore T, Panchal CJ, Russell I (1988) Factors that influence the ethanol tolerance of brewer's yeast strains during high gravity wort fermentations. MBAA Tech Quart 25:47–53
70. Suihko ML, Vilpola A, Linko M (1993) Pitching rate in high gravity brewing. J Inst Brew 99:341–346
71. Szajani B, Buzas Z, Dallmann K, Gimesi I, Krisch J, Tath M (1996) Continuous production of ethanol using yeast cells immobilized in preformed cellulose beads. Appl Microbiol Biotechnol 46:122–125
72. Theerarattananoon K, Lin YH, Peng DY (2008) Metabolic heat evolution of *Saccharomyces cerevisiae* grown under very-high-gravity conditions. Process Biochem 43:1253–1258
73. Thomas KC, Dhas A, Rossnagel BG, Ingledew WM (1995) Production of fuel alcohol from hull-less barley by very high gravity technology. Cereal Chem 72:360–364
74. Thomas KC, Hynes SH, Ingledew WM (1994) Effects of particulare materials and osmoprotectants on very-high-gravity ethanolic fermentation by *Saccharomyces cerevisiae*. Appl Environ Microbiol 60:1519–1524
75. Thomas KC, Hynes SH, Ingledew WM (1996) Practical and theoretical considerations in the production of high concentrations of alcohol by fermentation. Process Biochem 31:321–331
76. Thomas KC, Hynes SH, Jones AM, Ingledew WM (1993) Production of fuel alcohol from wheat by VHG technology: effect of sugar concentration and fermentation temperature. Appl Biochem Biotechnol 43:211–226
77. Thomas KC, Ingledew WM (1990) Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high gravity wheat mashes. Appl Environ Microbiol 56:2046–2050
78. Thomas KC, Ingledew WM (1992) Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes. J Ind Microbiol 10:61–68
79. Thomas KC, Ingledew WM (1995) Production of fuel alcohol from oats by fermentation. J Ind Microbiol 15:125–130
80. Verbelen PJ, Mulders SV, Saison D, Laere SV, Delvaux F, Delvaux FR (2008) Characteristics of high cell density fermentations with different lager yeast strains. J Inst Brew 114:127–133
81. Verbelen PJ, Schutter DPD, Delvaux F, Verstrepen KJ, Delvaux FR (2006) Immobilized yeast cell systems for continuous fermentation application. Biotechnol Lett 28:1515–1525
82. Viegas CA, Sá-Correia I, Novais JM (1985) Nutrient-enhanced production of remarkably high concentrations of ethanol by *Saccharomyces bayanus* through soy flour supplementation. Appl Environ Microbiol 50:1333–1335
83. Virkajarvi I, Vainikka M, Virtanen H, Home S (2002) Productivity of immobilized yeast reactors with very-high-gravity worts. J Am Soc Brew Chem 60:188–197
84. Wang FQ, Gao CJ, Yang CY, Xu P (2007) Optimization of an ethanol production medium in very high gravity fermentation. Biotechnol Lett 29:233–236
85. Wang S, Thomas KC, Sosulski K, Ingledew WM (1999) Grain pearling and very high gravity (VHG) fermentation technologies for fuel alcohol production from rye and triticale. Process Biochem 34:421–428
86. Wu X, Wang D, Bean SR, Wilson JP (2006) Ethanol production from pearl millet by using *Saccharomyces cerevisiae*. Paper No. 067077, 2006 ASABE annual international meeting, Portland, Oregon
87. Yingling B, Zongcheng Y, Honglin W, Li C (2011) Optimization of bioethanol production during simultaneous saccharification and fermentation in very high-gravity cassava mash. Antonie van Leeuwenhoek 99:329–339
88. Zhang L, Chen Q, Jin Y, Xue H, Guan J, Wang Z, Zhao H (2010) Energy-saving direct ethanol production from viscosity reduction mash of sweet potato at very high gravity (VHG). Fuel Process Technol 91:1845–1850
89. Zhang L, Zhao H, Gan M, Jin Y, Gao X, Chen Q, Guan J, Wang Z (2011) Application of simultaneous saccharification and fermentation (SSF) from viscosity reducing of raw sweet potato for bioethanol production at laboratory, pilot and industrial scales. Bioresour Technol 102:4573–4579