Volatile Esters and Fusel Alcohol Concentrations in Beer Optimized by Modulation of Main Fermentation Parameters in an Industrial Plant

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Keywords: response surface methodology, process optimization, manufacturing scale, industrial plant, sensory quality, volatile compounds, beer brewing

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Abstract: Contents of selected volatile esters and fusel alcohols and their relation to the sensory quality of a bottom-fermented lager beer fermented under high-gravity conditions (15.5 °P) were analyzed using response surface methodology (RSM, Box–Behnken design). The influence of various pitching rates (6–10 mln cells/mL), aeration levels (8–12 mgO_2/mL), times (4.5–13.5 h) of filling CCTs (cylindroconical fermentation tanks; 3850 hL), and fermentation temperatures (8.5–11.5 °C) on the contents of selected esters, as well as on concentrations of amyl alcohols and on the sum of higher alcohols in beer, was determined in a commercial brewery fermentation plant. Beers produced throughout the experiments met or exceeded all criteria established for a commercial, marketed beer. Statistical analyses of the results revealed that within the studied ranges of process parameters, models with diversified significance described the concentrations of volatiles in beer. The multiple response optimization procedure analyses showed that the values of process parameters that minimized higher alcohols in beer (97.9 mg/L) and maximized its ethyl acetate (22.0 mg/L) and isoamyl acetate (2.09 mg/L) contents, as well as maximized the sensory quality of beer, (66.4 pts) were the following: Pitching rate 10 mln cells per mL; fermentation temperature 11.5 °C; aeration level 8.8 mg/L; and CCT filling time 4.5 h.

Keywords: beer brewing; volatile compounds; sensory quality; industrial plant; manufacturing scale; process optimization; response surface methodology

1. Introduction

Beer forms a complex chemical matrix of components that result from numerous metabolic pathways and chemical reactions. He et al. [1] underlined the importance of interaction among various biosynthetic pathways during the fermentation process in a living yeast cell. The acceptable sensory properties of beer depend greatly on the control of the formation of desired volatile compounds during fermentation that is also important for achieving a repeatable and balanced composition of the finished product [2].

Many compounds contribute to the flavor and aroma of beer. Due to the low taste thresholds for some of these substances, supposedly insignificant variations in their concentrations may produce an entirely different flavor of the final beer. Thus, for pilsner beer, at least twenty compounds are recognized as being important. These substances include several esters, fusel alcohols, vicinal diketones, and organic sulfur compounds [3]. The latter two, which are present in a fresh, 'green' beer, are significantly reduced during lagering. The following compounds are considered the most important: Isoamyl alcohol, ethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate. However, it

would be an over-simplification to characterize the taste of beer just by the analytical determination of these five compounds. In practice, the flavor of one compound may easily be suppressed by high concentrations of other substances [4].

High-gravity brewing (HGB) employs worts at higher than normal concentrations (15–20 °P original gravity), and, therefore, to obtain beer of sales-gravity, dilution with deaerated water is required at a later stage of processing. HGB increases production without significant expansion of brewing, fermenting, and storage facilities. Therefore, the principal advantage of HGB is the more efficient use of the existing processes. The disadvantages of HGB include decreased foam stability of beer, a variety of stress effects on yeast, and problems with desirable flavor. Finally, difficulties encountered in HGB include the inability of yeast to completely utilize maltotriose, which is the most abundant fermentable sugar in the wort. This is particularly the case during beer production by continuous fermentation of wort under HGB conditions [5].

A factor of great importance in determining the flavor of beer is the composition of the wort. Small differences in wort composition can exert significant effects on the flavor of the resulting beer. Amino acids are among the wort components that may significantly influence beer flavor [6]. Volatile esters introduce fruity flavor notes and are considered highly positive flavor attributes of a fresh beer. Isoamyl acetate, for example, is a source of a banana-like flavor. However, during storage, the concentration of this ester can decrease to the levels that are well below its threshold level [7,8]. Among the volatiles, acetate esters like ethyl acetate, hexyl acetate, isoamyl acetate, and 2-phenylethyl acetate are recognized as a special and characteristic group of important flavor compounds of a lager beer [9].

Aroma-active esters are synthesized by yeast during fermentation in the intracellular space. It has been demonstrated that the esters partition between cells and fermenting medium depends mainly on yeast species and on the fermentation temperature. A higher proportion of esters that remain inside the cells are characteristic for lager yeasts (*Saccharomyces pastorianus, Saccharomyces carlsbergensis, Saccharomyces uvarum*) [10]. Moonjai with co-workers [11] reported that by the enrichment of wort with lipids, and also by increasing the level of wort aeration, synthesis of volatile esters was drastically reduced. The authors investigated the influence of the addition of unsaturated fatty acid (mainly linoleic acid) to harvested yeast prior to pitching, on the fermentation yield and on the synthesis of volatile flavor compounds, and found that the supplemented pitching yeast showed growth, attenuation, and ethanol formation profiles similar to those obtained with unsupplemented yeast in pre-aerated medium, which simulated the normal brewing practice. Compared to fermentations with unsaturated fatty acids added to the medium, the supplemented cropped yeast did not induce a reduction in acetate ester synthesis. Results indicated that the supplementation of cropped yeast with unsaturated fatty acids could be an interesting alternative to wort oxygenation to restore the optimal membrane fluidity of the yeast. Renger et al. [4], in turn, showed the importance of carbon dioxide for the growth and metabolism of fermenting yeasts. The excess of carbon dioxide had an inhibiting effect on the production of aroma compounds.

Amyl alcohol is reported to be the most present and quantitatively significant flavor compound of the higher alcohols group. Amyl, and its active isomer isoamyl alcohols, are, most of the time, described as amyl alcohols. These compounds affect beer drinkability as beer flavor is described by sensory analysis as "heavier" when the content of amyl alcohol increases. Another higher alcohol that affects the sensory quality of beer is isobutyl alcohol [10]. It may be stressed, therefore, that for the proper sensory characteristics of beer, the process optimization must ensure the maximization of ester concentration and, particularly, ethyl acetate that, in the right amounts, gives beer the fruity aroma impression, and also isoamyl acetate, which, in turn, produces a banana scent. On the other hand, however, the well-chosen process parameters should minimize the content of higher alcohols that generate the undesirable fragrances like alcohol, sweet, or a solvent scent.

Trelea et al. [12] presented an intriguing possibility of reducing the fermentation time without changing the aroma profile of beer volatile components. A few groups of researchers successfully developed predictive experimental models that incorporated process parameters for modulating the

biosynthesis of flavor-active compounds in the fermenting yeast cells [3,13–15], but none of these endeavors were performed utilizing a commercial fermentation plant.

Currently, statistical process control (SPC) techniques are increasingly being used in brewing. They allow controlled process maintenance with a very high repeatability of the process and the desired quality of the final beer. The most optimal fermentation process parameters developed by response surface methodology (RSM) guarantee the obtaining of a high stability of processes calculated by SPC. The production of beer on an industrial scale often employs constant values of temperature and regular pressure profiles. For many reasons, determination of optimal process parameters for a particular production plant is crucial. The key fermentation parameters of bottom-fermented lager beers brewed on an industrial scale can be successfully predicted, modulated, and controlled by applying the RSM methodology so that appropriate flavor and aroma compounds are synthesized at optimal concentrations.

The purpose of the current study was to apply the RSM methodology by developing empirical models to modulate the values of the fermentation temperature, pitching rate, aeration levels, and different times of filling the cylindroconical fermentation tanks in the industrial brewery, to control and predict the concentrations of volatile esters and fusel alcohols in a lager beer. The variations in the key process parameters had been limited, however, to be acceptable and ready for the market, and lager beer was also produced under experimental regimes. Yet, another aim of the study was to optimize the flavor and aroma compound concentrations to levels that ensure the best sensory quality of the final beer.

2. Materials and Methods

2.1. Experimental Setup

The process of beer fermentation and maturation was investigated in industrial cylindroconical fermentation tanks (CCT—cylindroconical fermentation tanks; gross volume 3850 hL with diameter 5.15 m and height 20 m) with different times (4.5–13.5 h) of filling CCTs. The experiments were carried out in a big commercial brewery in Poland. Each fermentation tank was filled with three brews (wort volume in every CCT—3090 hL). HGB worts (high gravity 15.5 °P) were prepared from the same batch of malt under identical technological conditions. A pilsener-type malt from two malt houses was used throughout the experiments. The process of infusion mashing-in took place within the standard scale of 60–76 °C. Sample collection started after filling the CCT and was continued during the following 18 days of the production cycle. Sampling from a tank was performed using a sampling device equipped with an installed small pump working in a closed loop system, which let us take samples of fermenting wort and of matured beer. Samples of beer were taken from CCTs at a point located above the conical part, 5 m from the bottom of the tank. *Saccharomyces pastorianus* brewers W34/70 yeast strain from Weihenstephan TUM was used for the fermentation. Total fermentation time lasted between 7 and 9 days depending on the selected fermentation parameters. The process of maturation was divided into two phases: Warm maturation and lagering. Yeast for experiments was cropped from CCTs during the 5th day of maturation (at temperature 13 °C). The warm maturation lasted 5 days at a temperature of 13 °C. After cropping, the yeast in the YST (yeast stored tank) was stored for a maximum of 4 days at temperatures of 1.3–1.8 °C with an overpressure of 0.05 bar. After cropping, the beer was cooled down to −0.7 °C (to phase of lagering). Yeast was pitched for first brews, using the fully automatic high-precision ABER system for rate control. Worts were aerated by compressed, sterile air during transfer to each CCT, with an identical intensity of 10 mg O_2/L wort. The processes of fermentation and maturation were carried out in the same technological conditions. The yeast growth ranged from volume factor 2.60 to 4.0 in relation to the initial pitching rate, and the Free Amino Acids (FAN) consumption varied from 112 to 144 mg/L.

2.2. Analytical Procedures

Extract marking was performed using an automatic wort and beer analyzer (Beer Analyzer DMA 4500+ Anton Paar, Graz, Austria), at 20 °C, and the specific weight was measured using an oscillating densitometer. The Tabarié formula was the basis for 'Alcolyzer' beer calculations [16].

Qualitative and quantitative analysis of volatile components (the identification was done on the basis of retention time) was performed using gas chromatograph GC 8000 (Fisons Instruments, Ipswich, UK) fitted with a flame ionization detector GC-FID and detector GC-ECD for detection of diacetyl, 2,3-pentanedione. The column temperature was kept at 45 °C for 10 min, increased to 120 °C at 5 °C/min, and then held at that temperature for 8 min, eventually being lowered to 45 °C at 15 °C/min. The temperature of the injection zone was fixed at 140 °C. The carrier gas was helium at a pressure of 65 kPa, with a flow of 4–6 mL/min. Injection of samples (0.75 mL) was performed with an HS-800 autosampler. The sample annealing temperature was 40 °C for 40 min. The temperature of the autosampler syringe was 60 °C. Concentrations were calculated using a quantitative computer program based on the calculated peak area. Selected components of beer were determined using surfaces under the curves produced relative to internal standards. The internal standard method involved introducing an internal standard to a test sample and determining the relationship between the peak area ratio of the test substance and the internal standard and the mass ratio of the test substance and the internal standard. The standards had to be well separated from other peaks in the sample and have a similar concentration as the substance to be determined. The sample of beer, with a volume of 2.5 mL, was placed in a vial and conditioned at 40 °C for 40 min to equilibrate the liquid and gas phase (head space method). The capillary column DB-WAX (dimensions: 60 m long, 0.53 mm internal diameter, and 1 μm thick) packed with polar polyethylene glycol was used for the separation. A mixture of 3-panthenol and n-butanol was used as an internal standard for the determination of esters, amyl alcohols, and the sum of higher alcohols. The chromatograph was calibrated once a month. Before and after each series of measurements, a comparative analysis was carried out with a beer sample used as a control (reference) batch.

2.3. Sensory Analysis

Sensory evaluation of bottling beer used a comparison test, with the test sample compared to the reference beer profile. The beer was tested in black glasses. Profile tests involved the evaluation of attributes of the beer, including fruity aroma esters, hops, bitterness, sulfur compounds, sweetness, acidity, fullness, balance, and flavor. The sensory analysis panel consisted of nine employees from the production, analysis, and technology departments whose standard job was to routinely assess the sensory quality of beer. The sample coding procedures used ensured objective evaluations. The sensory quality beer was evaluated using a gradation scale from 50 to 75 points where 70–75 points meant a very good or perfect example of beer; 65–69 points represented good, clean, and fresh beer; 60–64 points were allocated to neither good nor bad beer with low levels of undesirable flavors and aromas, 55–59 points - beer with one or more intense undesirable flavors and aromas, and 50–54 points represented a very bad - unfit for consumption, wrong product.

2.4. Statistical Analyses

Processing factors were tested using the Experimental Design Module of the Statgraphics Centurion XVII ver. 17.1.12 (Professional Edition statistical software, Statpoint Technologies, Inc., Warrenton, Virginia).

2.4.1. Optimization of The Volatiles and Sensory Quality of Beer

The influences of process parameters on the volatile concentrations and on the sensory quality of beer were studied using a fully randomized Box–Behnken design with four factors at three levels each and two blocks, including 3 centerpoints per block, which yielded 54 experimental runs and

38 degrees of freedom. There were two blocks with repetitions (all experiments were performed twice) serving as a block. The central composite design could not be used in this work, because it would generate experimental values of process parameters, resulting in the production of abnormal beer, not acceptable on the market. Table 1 illustrates the coded and actual values of the input variables (fermentation process parameters). Experimental worts were fermented using various pitching rates (6–10 mln cells/mL), aeration levels (8–12 mg/mL), times (4.5–13.5 h) of filling CCTs (cylindroconical fermentation tanks; 3850 hL), and fermentation temperatures (8.5–11.5 °C). The manufactured beer was then subjected to the volatile concentration and sensory quality analyses (measured responses). The relationship between the measured exposures and fermentation process parameters was expressed using second-order polynomial equations:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_{11} {x_1}^2 + \beta_{22} {x_2}^2 + \beta_{33} {x_3}^2 + \beta_{44} {x_4}^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{14} x_1 x_4 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4 + \gamma$$

where y is a volatile concentration or sensory quality; x_1 is the pitching rate (mln yeast cells/mL of wort); x_2 is the fermentation temperature (°C); x_3 is the aeration level (mg O_2/L); x_4 is the total time used for CTT filling (h); β_0 is the intercept coefficient; $\beta_{1–4}$ are the linear coefficients; β_{11}, β_{22}, β_{33}, and β_{44} are the quadratic coefficients; and β_{12}, β_{13}, β_{23}, β_{14}, β_{24}, and β_{34} are the interaction coefficients, whereas γ is the block effect.

Table 1. Coded and actual values of the variables for the Box–Behenken design.

Independent Variables	Units	Symbol	Coded Levels		
			−1	0	+1
Pitching rate	Mln cells/mL	x_1	6	8	10
Fermentation temperature	°C	x_2	8.5	10	11.5
Aeration level	mg/L	x_3	8	10	12
Total time of CCT filling	h	x_4	4.5	9	13.5

The established models were subjected to ANOVA and Pareto chart (data not shown) analyses, and the non-significant ($p > 0.05$) components were removed from the models. To evaluate the statistical significance of the second-order polynomial model, the coefficient of determination (R^2) and the probability of the lack-of-fit values were calculated.

2.4.2. Multiple Response Optimization Procedures

The module Multiple Response Analysis of the Statgraphics Centurion XVII ver. 17.1.12 (Professional Edition statistical software, Statpoint Technologies, Inc., Warrenton, Virginia) was used to establish the values of technological parameters that simultaneously optimized the content of a few measured responses.

3. Results and Discussion

3.1. Model Fitting

A significant influence of the process parameters with the coefficient of determination exceeding 0.70 on the ethyl acetate, isoamyl acetate, higher alcohols, amyl alcohols, and isobutanol concentrations was observed, but in the case of methanol, 1-propanol, ethyl formate, ethyl capronate, and ethyl propionate, lower values of R^2 were calculated within the studied ranges of the pitching rate, fermentation temperature, aeration level, and times of CTT filling (Table 2). The volatiles with a determination coefficient lower than 0.60 were excluded from further optimization.

Table 2. Analysis of variance of volatile esters and fusel alcohols: Significance of model components and assessment of adequacy of the models.

Dependent Parameter	Analysis of Variance							
	R^2	Lack-of-Fit	x_1	x_2	x_3	x_4	Significant Components of the Model	
	Probability							
Higher alcohols	0.91	0.932	0.022	0.0193	Ns	0.0003	0.0473	x_3x_4
Amyl alcohols	0.91	0.808	0.027	0.025	Ns	0.0001	0.0473	x_3x_4
Methanol	0.53	0.578	0.0307	ns	Ns	0.0400	0.0327	blocks
Isobutanol	0.68	0.358	0.0039	0.0461	Ns	0.035	0.0061	x_1^2
1-propanol	0.30	0.000	0.0001	0.027	Ns	0.0001	0.0001	x_1^2
Ethyl acetate	0.89	0.967	0.0032	0.0019	Ns	ns	-	-
Isoamyl acetate	0.69	0.953	0.0298	0.0054	Ns	ns	-	-
Ethyl formiate	0.62	0.813	0.0356	ns	Ns	ns	0.0488	x_1x_4
Ethyl capronate	0.56	0.0967	ns	ns	Ns	0.0247	-	-
Ethyl propionate	0.39	0.450	0.0179	0.0131	Ns	ns	-	-
Sensory analysis	0.71	0.0951	0.0213	0.0012	0.0089	0.0272	0.0021	x_1^2
							0.0474	x_1x_2
							0.0299	x_1x_4
							0.0040	x_2^2
							0.0021	x_2x_3
							0.0102	x_3^2
							0.0384	x_4^2

3.2. Polynomial Equations for the Measured Responses

Ethyl acetate

Table 3 shows the analysis of variance for ethyl acetate content in matured beer after removing insignificant components from the model. The relationship between the only two significant factors and the predicted responses of ethyl acetate concentrations was modeled as follows:

$$y_1 = -1.295 + 0.850\, x_1 + 1.293\, x_2 \quad (1)$$

where y_1 denotes ethyl acetate concentration.

Table 3. Analysis of variance: the empirical model for predicting ethyl acetate.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*p*-Value
x_1	69.2920	1	69.2920	54.14	0.0000
x_2	90.2682	1	90.2682	70.53	0.0000
blocks	0.0308	1	0.0308	0.02	0.8776
Lack-of-fit	24.1341	14	1.7239	1.35	0.2295
Pure error	46.0771	36	1.2799		
Total (correlation)	229.8020	53			

As shown in Table 3, small *p* values (<0.05) were observed for the constant, a linear term x_1, and linear term x_2, indicating that only pitching rate and fermentation temperature were the crucial process parameters affecting amounts of ethyl acetate in beer. The subsequent application of the established model to predict the process parameters optimal for ethyl acetate biosynthesis using the Optimize Response module revealed that over the studied range of process parameters, to achieve maximized ethyl acetate concentrations, the values of process parameters should be set to $x_1 = 10$, $x_2 = 11.5$, $x_3 = 10$, and $x_4 = 9$. Under these conditions, 22.07 mg/L of ethyl acetate was predicted to be synthesized, a value that can be perceived as an acceptable level of this volatile in beer. Verstrepen with co-workers [17],

who reviewed the literature on wort specific gravity and wort sugar profiles, reported the problem of overproduction of acetate esters in the HGB.

There seems to be a general consensus in the literature, however, that with growing fermentation temperature, the biosynthesis of acetate esters is enhanced [12,15,17]. In the work of Lee and Davis [18], a 10-fold increase in pitching rate caused a two-fold increase in the concentration of ethyl acetate in beer that seems to be in line with the results of our studies. The data presented by other researchers, however, did not confirm these observations. Verbelen et al. [19] did not show changes in the content of ethyl acetate with increasing amounts of inoculum from 10 to 120 mln cells/mL. Similar findings were provided by Erten [20] who reported that, as a result of increasing yeast pitching rate from 1×10^7 to 1×10^8 cells/mL, the concentration of that volatile remained within the range of 13–14 mg/L and was not statistically significant. These discrepancies might mainly be attributed to differences in working volumes of the experimental fermentors used and, consequently, to differences in hydrostatic pressure that affected the metabolism of yeast.

Isoamyl acetate

Similarly to ethyl acetate, the analysis of variance for isoamyl acetate concentrations in beer revealed that yeast pitching rate and fermentation temperature significantly affected the biosynthesis of this ester (Table 4).

Table 4. Analysis of variance: the empirical model for predicting isoamyl acetate.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*p*-Value
x_1	0.7245	1	0.7245	22.50	0.0000
x_2	1.9982	1	1.9982	62.05	0.0000
Blocks	0.0011	1	0.0011	0.03	0.8566
Lack-of-fit	0.5608	14	0.0400	1.24	0.2885
Pure error	1.1592	36	0.0322		
Total (correlation)	4.4437	53			

Linear in nature, a simple equation that links amounts of isoamyl acetate with process parameters was the following:

$$y_2 = -0.857 + 0.0869\, x_1 + 0.192\, x_2 \quad (2)$$

where y_2 denotes isoamyl acetate concentration in mg/L.

Maximizing the predicted isoamyl acetate concentrations by means of the Optimize Response module allowed us to achieve 2.22 mg of that volatile per liter of beer. The values of process parameters set at $x_1 = 10$, $x_2 = 11.5$, $x_3 = 10$, and $x_4 = 9$ were calculated as optimal for the highest ethyl acetate concentrations. Nakatani with co-workers [21] reported double the rate of isoamyl acetate synthesis, which resulted from rising fermentation temperature from 10 to 15 °C. Lima et al. [15] and Brown and Hammond [13] observed similar tendencies. In yet another study, Saerens et al. [22] demonstrated that increasing the fermentation temperature by 3 °C resulted in a 50% increase in the concentration of isoamyl acetate. Our studies also suggest that the initial rate of yeast addition to wort was positively related to concentrations of this volatile in beer. Verbelen with co-workers [19] provided evidence of the significance of such a correlation within the range of yeast concentrations from 10 to 40 mln cells/mL.

Amyl alcohols and the sum of higher alcohols

The analysis of variance for amyl alcohol concentrations in beer is given in Table 5, whereas similar analysis for the sum of higher alcohols is shown in Table 6.

Table 5. Analysis of variance: the empirical model for predicting amyl alcohols.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*p*-Value
x_1	209.5690	1	209.5690	43.40	0.0027
x_2	218.6480	1	218.6480	45.28	0.0025
x_3	0.4817	1	0.4817	0.10	0.7679
x_4	968.5020	1	68.5020	200.56	0.0001
x_3 x_4	38.6760	1	38.6760	8.01	0.0473
Blocks	7.7521	1	7.7521	1.61	0.2739
Lack-of-fit	129.9700	43	3.0226	0.63	0.8077
Pure error	19.3162	4	4.8291		
Total (correlation)	1592.9100	53			

Table 6. Analysis of variance: the empirical model for predicting the sum of higher alcohols.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*p*-Value
x_1	496.4050	1	496.4050	49.00	0.0022
x_2	145.2880	1	145.2880	14.34	0.0193
x_3	2420	1	4.2420	0.42	0.5528
x_4	1515.1100	1	1515.1100	149.55	0.0003
x_3 x_4	81.1538	1	81.1538	8.01	0.0473
Blocks	30.1953	1	30.1953	2.98	0.1594
Lack-of-fit	250.8160	43	5.8329	0.58	0.8406
Pure error	40.5250	4	10.1312		
Total (correlation)	2563.7400	53			

The striking similarity of significant components in models predicting concentrations of amyl alcohols and the sum of higher alcohols suggested the important role of the volatile amyl alcohols in these beer flavor components. With the exception of aeration rate, all other process parameters of beer fermentation significantly modulated the concentrations of volatile alcohols in beer. As shown in Tables 5 and 6, among process parameters, p values < 0.05 were observed for linear terms of x_1, x_2, and x_4, as well as for interaction term x_3 x_4.

The polynomial functions for amyl alcohols:

$$y_3 = 9.498 + 1.478x_1 + 2.012x_2 + 2.270x_3 + 3.854x_4 - 0.244x_3x_4 \quad (3)$$

and for the sum of higher alcohols:

$$y_4 = 18.116 + 2.274x_1 + 1.640x_2 + 3.395x_3 + 5.305x_4 - 0.354x_3x_4 \quad (4)$$

allowed us to recognize the time of CTT filling as the most significant factor. The established models were subsequently applied to predict the optimal process parameters for minimizing higher alcohol concentrations in beer. The value of each of the process parameters when kept at the lowest levels guaranteed values as low as 62.30 mg/L of amyl alcohols, and 85.6 mg/L of higher alcohols in beer.

There have been multiple experiments undertaken to assess the impact of process parameters on the higher alcohol concentration in fermenting wort. Jones with co-workers [23] showed that the content of fusel alcohols like isobutanol, isoamyl alcohol, and 1-propanol was only slightly higher when additional amounts of oxygen were applied 12 h post-inoculation. Erten et al. [20] reported that a higher pitching rate led to an increase in the concentration of isobutanol but also to a decrease in the content of active amyl alcohols, like 2-methyl-1-butanol. Jones with co-workers [23], however, reported that the concentrations of 1-propanol increased with higher yeast pitching rates. The experiments conducted by Lima et al. [15] also confirmed that increased pitching rate from 15 to 22 mln cells/mL caused a rise in the concentration of 1-propanol. There seems to be a general agreement in the literature

that biosynthesis of fusel alcohols is positively related to the temperature of fermentation [3,13,24,25]. All these reports seem to be in good agreement with the observations of this study.

Sensory quality

The proper and unchanging sensory quality of beer is one of the most important problems in brewing, particularly in the HGB method. The flavor stability and repeatability of good sensory properties of beer were maintained throughout the current study (65.7 to 66.7 points).

Table 7 lists significant components of the model that relates the sensory quality of beer to the values of process parameters applied during fermentation. A few two-factor interaction terms were found insignificant in the original model, and each of the process parameters had a significant quadratic term. Fermentation temperature appeared to be the key factor influencing the sensory characteristic of lager beer. Both a linear and quadratic component of the temperature, as well as a significant interaction of the temperature with aeration level, were among the main determinants of the sensory quality. There was also a significant negative effect of the pitching rate and a positive effect of its quadratic component. The complete polynomial equation that related the sensory quality of beer to the values of process parameters was the following:

$$y_5 = 61.255 - 0.691\ x_1 + 1.693\ x_2 - 0.148\ x_3 + 0.0370\ x_4 + 0.0828\ {x_1}^2 - 0.0542\ x_1\ x_2 - 0.0167 x_1\ x_4 - 0.115\ {x_2}^2 + 0.121\ x_2\ x_4 - 0.057\ {x_3}^2 + 0.0083\ {x_4}^2 \quad (5)$$

where y_6 denotes the sensory quality of beer (in points).

Table 7. Analysis of variance: the empirical model predicting the sensory quality of beer.

Source	Sum of Squares	Df	Mean Square	F-Ratio	*p*-Value
x_1	0.3267	1	0.3267	17.40	0.0145
x_2	1.4259	1	1.4259	74.40	0.0010
x_3	0.6176	1	0.6176	32.22	0.0048
x_4	0.2017	1	0.2017	10.52	0.0316
${x_1}^2$	1.1704	1	1.1704	61.07	0.0014
$x_1\ x_2$	0.2113	1	0.2113	11.02	0.0294
$x_1\ x_4$	0.1800	1	0.1800	9.39	0.0375
${x_2}^2$	0.7177	1	0.7177	37.44	0.0036
$x_2\ x_3$	1.0513	1	1.0513	54.85	0.0018
${x_3}^2$	0.5551	1	0.5551	28.96	0.0058
${x_4}^2$	0.3038	1	0.3038	15.85	0.0164
Blocks	0.0007	1	0.0007	0.03	0.8648
Lack-of-fit	3.2974	37	0.0891	4.65	0.0716
Pure error	0.09	4	0.0225		
Total (correlation)	11.457	53			

It appears that due to the significant interaction term (x_2x_3), the level of wort aeration determined the character of changes in the sensory quality of beer, which resulted from different fermentation temperatures. At high aeration level, the raising temperature enhanced the sensory quality of beer almost linearly, but at a low aeration rate, there was a plateau and then a decline in beer quality. When Equation (5) was applied for maximizing sensory quality, a lager with excellent sensory quality (67 points) was predicted when the pitching rate was set at the low level and all the other parameters were maintained at their respective high values. In one-factor experiments, a direct, positive relationship between fermentation temperature and the sensory quality of beer was reported by Brown and Hammond [13]. The direct connection of a correct wort oxygenation and the sensory quality of beer that was observed in this study had also been reported earlier [19].

3.3. Multiple Response Optimization Procedures

Effects of process parameters on the multiple measured exposures were assessed by the Multiple Response Optimization procedure of the Statgraphics software.

At first, combined maximization of volatile estes (ethyl acetate and isoamyl acetate) concentrations was undertaken. The next optimization that involved all volatile components comprised the maximization of ester concentrations and simultaneous minimization of volatile alcohols, i.e., isobutanol and the sum of higher alcohols. The final optimization ("optimize all") also included the maximization of beer sensory quality. A comparison of results from the single response optimizations described earlier to those originating from the multiple response optimization procedure, as well as the predicted values for each of the measured beer volatile and the sensory quality of beer, is presented in the Table 8.

Table 8. Values of process parameters that optimized ethyl acetate, isoamyl acetate, isobutanol concentration, and the sensory quality of beer with corresponding predicted values, and the multiple response optimization: Esters = ethyl acetate + isoamyl acetate; alcohols = butanol + higher alcohols, volatiles = esters + alcohols, and all = esters + alcohols + sensory quality.

Technological Parameters	**Levels**		**Optimum/Goal**								
			EtAcet	**IsAc**	**Esters**	**Isobutanol**	**HA**	**Alcohols**	**Sensory**	**Volatiles**	**All**
	−1	**+1**	**Maximize**			**Minimize**			**Maximize**	**Optimize**	**Optimize**
Pitching rate (mln cells/mL)	6.0	10.0	10.0	10.0	10.0	6.0	6.1	6.0	6.0	10.0	10.0
Temperature of fermentation (°C)	8.5	11.5	11.5	11.5	11.5	11.3	8.8	9.6	11.5	11.5	11.5
Wort aeration level (mg/L)	8.0	12.0	10.0	9.6	10.0	8.0	8.0	8.0	11.1	8.1	8.8
Total filling time CCTs (h)	4.5	13.5	9.0	13.5	13.5	4.5	4.5	4.6	13.5	4.7	4.5
Volatiles/Sensory			**Predicted Values**								
Ethyl acetate (EtAcet; mg/L)			22.1		22.1					22.1	22.0
Isoamyl acetate (IsAc; mg/L)				2.34	2.34					2.1	2.09
Isobutanol (mg/L)						11.8		12.6		12.6	12.9
Higher alcohols (HA; mg/L)							83.5	85.2		97.4	97.9
Sensory quality (pts)									67		66.4

As evidenced in Table 8, the fermentation temperature set at the high level (11.5 °C; +1) optimized the concentrations of volatile esters, the sum of volatile substances, and the sensory quality of beer and was also required for the overall optimization. The low pitching rate (−1) guaranteed the highest sensory quality, but the high pitching rate (+1) was necessary for both optimal volatile concentrations, and for the general optimization. The time of CTT filling was calculated to be set at 13.5 h (+1) for optimal volatile ester concentration and for good sensory quality of beer; however, for the overall optimization, which, in addition to sensory quality, comprised both volatile esters and volatile alcohols, the short time of CTT filling (4.5 h; −1) was simulated. With the exception of fermentation temperature, the levels of process parameters that were simulated to guarantee optimal concentrations of volatile compounds in beer differed from those that were calculated as optimal for the sensory quality of beer. This finding clearly suggests that volatiles other than esters and fusel alcohols play a predominant role in determining the sensory quality of beer. In our previous study [26], the process parameters that optimized acetaldehyde and DMS concentrations were the same as those that maximized the sensory quality of beer. Volatile compounds in fermented beverages have been found to strongly influence the sensory characteristics of the product, and, therefore, their identification and optimization are of utmost importance for understanding the relationships among different process parameters and chemical composition, as well as for maintaining and for further enhancements in the product quality [27].

There have been attempts to optimize process parameters in high-gravity brewing fermentations performed in a laboratory or in a pilot plant by varying values of the most important process parameters in statistically designed experiments [23]. None of these endeavors, however, have aimed to provide a direct relationship between results of such optimization and the sensory quality of beer produced on an industrial scale.

4. Conclusions

The multiple response optimization procedure of the Statgraphics software allowed us to find levels of the process parameters that optimized concentrations of esters, higher alcohols, and the sensory quality of a lager beer. The values of process parameters that maximized the concentrations of volatile esters differed from those that minimized the concentrations of higher alcohols. The simultaneous optimization of volatile compounds and the sensory quality of beer yielded overall values of process parameters: Pitching rate—10 mln cells per mL; fermentation temperature—11.5 °C; aeration level—8.8 mgO_2/L, and time of filling CCTs—4.5 h. These levels may be perceived as a result of a compromise between optimal volatile esters and higher alcohol concentrations. It is suggested that volatiles other than esters and fusel alcohols must have played a predominant role in determining the sensory quality of beer. We suggest that the RSM modeling can be successfully used for prediction and control of important process parameters of fermentation performed in an industrial plant to have desirable taste and aroma of bottom-fermented lager beers.

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