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MAXIMIZING TOTAL PROTEIN EXTRACTION FROM SPENT BREWER'S YEAST USING HIGH-PRESSURE HOMOGENIZATION

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Abstract

Large quantities of spent brewer's yeast are generated as a by-product during beer production, representing a cost-effective, nutrient-rich substrate. Spent brewer's yeast is especially rich in protein and several methods have been tested in order to maximize the protein extraction and reduce the costs of this extraction. Here we present the optimization of total protein extraction from brewer's spent yeast by design of experiments (DOE) using a high-pressure piston homogenizer. The yeast was pre-treated with a β-glucanase for one hour at 50°C. A full factorial design with two levels, four factors (yeast amount, pressure, number of passes, enzyme concentration) was used. The model and the data indicated that two parameters (pressure, enzyme concentration) were highly significant ($p < 0.05$), while the other two parameters (yeast amount, number of passes) were moderately significant and not significant, respectively. The interaction between pressure and enzyme concentration was also marginally significant. Our optimization indicates that efficient extraction of proteins from brewer's spent yeast could be obtained and up-scaled with minimal costs.

Key words*: total yeast protein extract, design of experiments, piston homogenizer, enzymatic treatment.*

INTRODUCTION

Beer is a staple drink in many countries around the world. However, this high demand generate pressure on the brewing industry to produce more and more beer, which in turn creates large amounts of residues that are usually vastly under-utilized.

The industrial process of obtaining beer, as described by Carlsberg uses several simple ingredients: water, malt, hops and yeast. The first step in the process involves turning the barley into malt, then transforming the starches in the malt into fermentable sugars. Hops are added for flavour and the mixture is boiled for an hour. Afterwards, the liquid fraction is cooled and yeast is added to start the fermentation process which can last between 7 and 14 days (Carlsberg).

The driving force behind beer fermentation is the yeast, *Saccharomyces pastorianus*, which was specially selected to produce alcoholic beverages with a low alcohol content. This type of yeast is a bottom fermenting lager-type, which means that it thrives at colder temperatures, as opposed to top fermenting ale-types of yeast (such as *Saccharomyces cerevisiae*) which prefer a warmer environment. There are also differences in the taste profile of beer obtained through these two methods, lager types boasting a crisp and "cleaner" taste compared to the bitter ale-types (Bonatto, 2021). The former seems to have captured the hearts and taste buds of most consumers, with ale types having a smaller, but loyal fan base. A case in point is represented by the Romanian beer industry. An analysis from 2012 (Dobre-Baron, 2012) reveals the fact that the beer Romanians consume is 99% locally sourced with a staggering percent of it being lager-type. This amounts to an average production volume of approximately 15 million

hectolitres. For each hectolitre of beer produced, an average 3 kg of spent brewer's yeast (SBY) is produced, which in time amounts to more than 44000 tonnes of spent yeast (San Martin et al., 2018).

However, in the last years, there have been efforts to valorise this yeast through different methods. Due to its high protein content (between 45 and 60% w/w) yeast seems to be a perfect protein source and is generally regarded as safe for human consumption. Still, yeast protein extracts contain between 6 and 15% nucleic acids which in humans cause an elevation of uric acid levels in the blood (Podpora et al., 2016). Thus, spent brewer's yeast has been used as a cheap source of feed for livestock or just disposed of as a waste into the environment (Puligundla et al., 2020). Spent brewer's yeast cannot be consumed in the form released from the brewery, so the best solution is the extraction of economically valuable compounds such as yeast cell walls, aroma compounds etc. Yeast cell walls contain fibres, mainly β-glucans and α-mannans, which serve an important role in nutrition. The presence of bioactive peptides as well as other functional components, such as carotenoids, oligosaccharides, polyphenols make yeast a golden mine for nutrition (Rai et al., 2019). While they represent the basics of good nutrition, they could also be used for creating healthier crops and enhancing plant nutrition through their effects on plant metabolism.

One important trend in the last years has been the use of natural, agricultural inputs, included in the category of plant biostimulants. The EC 2019/1009 regulation has opened the market as well as given a vote of confidence to plant biostimulants while also offering some guidelines towards regulating the claims and the contaminants (European Union, 2019). Plant biostimulants are considered to be any agricultural inputs, that brings an improvement to plant nutrition, crop health or resistance to biotic or abiotic stress, however they do not fit under the umbrella of fertilizers, pesticides or biocontrol agents (du Jardin, 2015).

Type-wise, the plant biostimulants can be microbial or non-microbial. The non-microbial ones are further on split into other categories, based on the chemical composition. The most abundant biostimulants on the market are represented by humic and fulvic acids, followed by seaweed extracts and finally plant and other types of extracts among which the most important to mention are amino acids and protein hydrolysates. Protein hydrolysates can be obtained from different sources with high protein content, one such source being industrial waste from the brewing industry. The usual method of extraction involves a process called autolysis which consists of heating the yeast slurry in order to activate the intra-cellular enzymes and proteases to destroy the membrane and the cell wall and thus, release the cellular content (Takalloo et al., 2020). However, this process can be approached in a different manner by using pressure and enzymatic pre-treatment to elevate the efficiency of the process.

There is little study in the field of high pressure homogenization and enzyme assisted extraction of proteins from yeast. Most of the previously published data used high pressure homogenisation as a pre-treatment for yeast autolysis (Baldwin & Robinson, 1990; Dimopoulos et al., 2020; Verduyn et al., 1999), but there is little knowledge on the optimisation of the high-pressure homogenisation parameters for the purpose of obtaining yeast extract high in protein content. It is also worth mentioning that these studies used different intervals for the parameters, which could influence the protein yield.

The aim of this study was to develop an improved method of industrial processing of spent brewer's yeast by using a multi-factor experimental design and to determine which parameters should be carefully controlled to release high quantity of protein from the treated yeast and to allow separation of the yeast cell wall. The final goal for this yeast cell breakage separation of the two components, yeast extract and yeast cell wall, is related to agricultural applications of both resulted components.

MATERIALS AND METHODS

The spent brewer's yeast was supplied by our partners AGSIRA SRL and its provenience is from one of the beer factories in Romania. All used yeast was lager-type. The yeast was supplied in the dry-compacted form. The spent yeast slurry was previously dried, by using a

double-drum dryer (T9/30, Gouda, Waddinxveen, Netherlands), operated at 140°C and 3 rpm.

The dried yeast was resuspended in deionized water and pre-treated with a commercial β-glucanase preparation (VinoTaste® Pro, Novozymes, Bagsværd, Denmark) at 50°C for one hour under stirring. The commercial enzyme preparation include also enzymes with pectinolitic activity (Averilla et al., 2019). However, pectinases are not active against plant cell wall.

Afterwards, the mixture was homogenized using a Lab Homogenizer Panda PLUS 2000 (GEA Niro Soavi, Parma, Italy), applying different experimental settings generated by the design of experiment described below. The experimental design was created using a full factorial design with two levels and four factors, namely: yeast amount (as percent concentration), homogenizer pressure applied, number of passes and enzyme concentration. The two levels were 10 and 20% for yeast, 1000 and 2000 bar for pressure, 3 and 7 number of passes and 0.1 and 0.3 mg/L for the enzyme concentration. Three centre points were added to estimate the standard error of the design space.

The yeast extracts were stored overnight at 4°C to favour sedimentation of cell walls and remaining yeast. Further separation of the two phases was done through centrifugation. The supernatant was tested for protein concentration using a modified biuret method (Gornall et al., 1949).

The protein extract was spray-dried (by using a B-290 Mini Spray Dryer, Büchi, Flawil, Switzerland) to prevent spoilage and stored for further applications. The precipitate containing the yeast cell walls was freeze-dried and stored for further analysis.

The data were analysed using Design-Expert 11 software (Sta-Ease, Minneapolis, MN). Statistical significance of the terms was determined by ANOVA (analysis of variance).

RESULTS AND DISCUSSIONS

The experimental setup that was used, involves a set of conditions which were randomized to gain as much information concerning a possible model, from a lower number of experiments. The experimental design is presented in Table 1 and shows the combination of conditions required for each of the considered factors, in each of the 19 experimental points used into this optimization study.

Table 1. Parameters of the experimental setup

Exp. no.	Yeast concentration (%) (A)	Enzymatic pre-treatment (g enzyme per L) (B)	No. of passes (C)	Homogenizer pressure (bar) (D)
D1	10	0,1	3	1000
D2	20	0,1	3	1000
D3	10	0,3	3	1000
D4	20	0,3	3	1000
D5	10	0,1	7	1000
D6	20	0,1	7	1000
D7	10	0,3	7	1000
D8	20	0,3	7	1000
D9	10	0,1	3	2000
D10	20	0,1	3	2000
D11	10	0,3	3	2000
D12	20	0,3	3	2000
D13	10	0,1	7	2000
D14	20	0,1	7	2000
D15	10	0,3	7	2000
D16	20	0,3	7	2000
D17	15	0,2	5	1500
D18	15	0,2	5	1500
D19	15	0,2	5	1500

An experimental design was used to explore the effects and the relationship between several variables and the effect they have on the response, as well as optimise the response to certain levels presenting interest. The samples were analysed using a modified biuret method as presented by Gornall and his collaborators (Gornall et al., 1949).

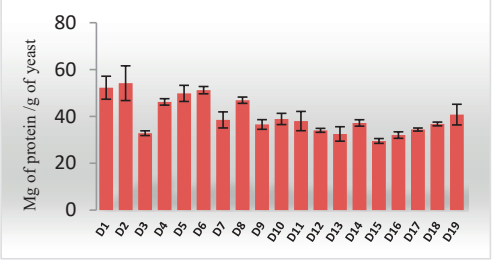

Figure 1. Protein yield measured through Biuret assay on the soluble protein obtained through enzymatic and pressure treatment of spent brewer's yeast. The bars represent the standard error

The data described in Figure 1 represent the primary response variable which was used to further investigate the relationship between the varied parameters.
The desired response is represented by the highest protein yield per gram of yeast used. The data indicates that this is achieved for the conditions applied to sample D2. Statistical significance of the terms was assessed by ANOVA (analysis of variance) using Design-Expert 11. The most appropriate model was obtained by checking iteratively the p-values of the terms starting from the design model (4 main terms, 6 secondary interaction terms and higher-level interaction terms).
Table 2 shows the results for the best-fitting model involving significant terms and marginally significant terms.

Table 2. Statistical analysis of data generated by the most appropriate model

Source	Sum of Squares	Mean Square	p-value	Significance
Model	961.82	137.40	0.0006	**S**
A-Yeast concentration	67.21	67.21	0.0525	**MS**
B-Enzymatic Pre-treatment	174.74	174.74	0.0049	**S**
D-Pressure	578.50	578.50	< 0.0001	**S**
AB	2.34	2.34	0.6926	**NS**
AD	26.37	26.37	0.2006	**NS**
BD	60.55	60.55	0.0635	**MS**
ABD	52.11	52.11	0.0820	**MS**
Residual	156.50	14.23		
Lack of Fit	113.19	12.58	0.7673	**NS**
Pure Error	43.32	21.66		
Cor Total	1118.32			

In the significance column, S = the parameter is significant, MS = the parameter is marginally significant, NS = the parameter lacks significance in the model

From the analysed parameters, some presented higher significance ($p<0.05$) while others were only marginally significant. The primary factors namely enzymatic pre-treatment (B) and pressure (D) presented significance, while yeast concentration (A) was only marginally significant ($p = 0.052$). The number of passes (C) did not present significance in this model. The secondary and tertiary interaction factors were also taken into consideration. The secondary interaction factor BD as well as the tertiary interaction factor ABD were the only ones within the marginally significant group, with $p_{BD} = 0.063$ and $p_{ABD} = 0.082$. Overall, the model had a high significance, with a value of $p = 0.006$.
Pareto charts are used to delimitate the most important factors and place them in a descending order. In our case, the Pareto chart supports the choice of parameters to include in the model, as it can be seen in Figure 2. The factor with the highest significance, pressure, drives both the values for the Bonferroni limit and that of the t-test upwards, compared to those corresponding to the marginally significant terms.
Thus, both primary factors with significance, enzymatic pre-treatment and pressure may influence the quantity of extracted protein, and the marginal significance of their interaction factor may point at a synergic effect of these two parameters.

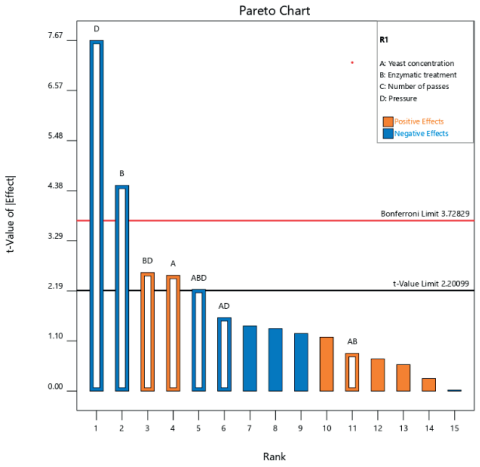

Figure 2. Pareto chart depicting the importance of the parameters in descending order. The parameters are labelled using their codification mentioned in the ANOVA analysis

The yeast concentration, which presents marginally significant p-values tends to explain the variation of data, but in a lesser degree than the other primary factors.
The equation characterising the model is described below with coded values for the factors:

$$Prot.\ Yield\ \left(\frac{mg}{g}\right) = 41.47 + 2.05 \cdot A - \\ - 3.30 \cdot B - 6.01 \cdot D + 0.38 \cdot AB - 1.28 \cdot AD + \\ 1.95 \cdot BD - 1.80 \cdot ABD, \quad (1)$$

Where:
Prot. Yield = the main response (the protein yield of the extraction)
A = Yeast concentration
B = Enzymatic Pre-treatment
D = Pressure
The combination factors = the secondary interaction factor between A and B, A and D and B and D; the tertiary interaction factor between A, B and D.

The equation (1) provides some interesting insights into how each parameter affects the protein yield. The negative terms have a negative impact, their increase leading to a decrease of the protein yield, at least in the case of the primary significant parameters, the most poignant negative effect being that of pressure, closely followed by that of enzymatic pre-treatment.
The adequacy of a model's ability to describe the interactions between the parameters is measured through the lack of fit. In our case, this is not significant which supports the fact that the model is well suited to describe the interactions of the parameters (StatEase, n.d.).
To support this, we need to take into consideration the $\mathbf{R^2}$ and the adjusted $\mathbf{R^2}$ values which indicate the amount of variation around the mean explained by the model. It is considered as a rule of thumb that the closer this value is to 1, the better the model. In our case the $\mathbf{R^2}$ = 0.86 and **adjusted** $\mathbf{R^2}$ = 0.77, which also points to the fact that the model is fitting.
A set of 3D surface plots (Figure 3) were generated using equation (1) and yeast concentration and enzymatic treatment as axis for the plots. Pressure was varied between the low level (A) and the high level (B), to observe the aspect of the curves.

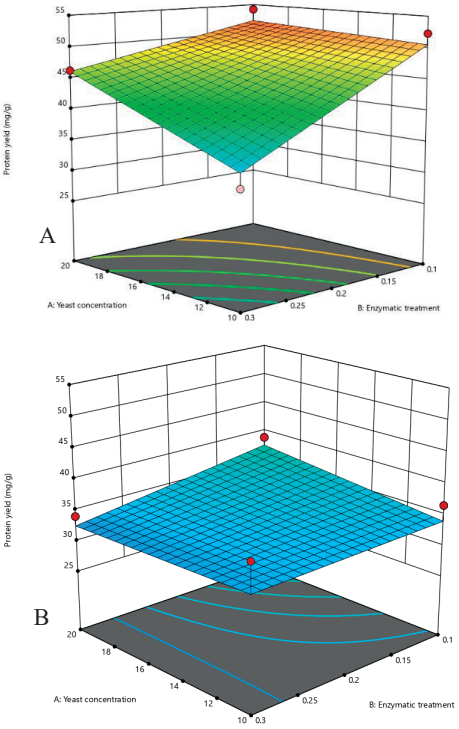

Figure 3. 3D plots of protein yield, as a function of yeast concentration and enzymatic treatment, when the pressure is 1000 bars (A) and when the pressure is 2000 bars (B). The number of passes was kept constant for the two graphs at a value of 3

In terms of soluble protein yield, our results indicate the fact that in the case of this experimental setup, higher yield was obtained for lower values for both pressure and enzyme concentration and using more pressure and more enzyme does not bring any improvement to the process.

Table 3. Highest and lowest concentrations of soluble proteins as measured through the Biuret reaction

Sample	Concentration of soluble protein (mg/mL)
D2	11.22
D15	2.95

One study (Dimopoulos et al., 2020) indicates a corelation between the obtained soluble protein concentration and combining different

approaches to this issue. Our results indicate the fact that our approach seems to be more efficient, the values exceeding by far the values mentioned in literature.

There is another matter that might be useful in understanding what happens with the intensification of processes. Higher shear force, driven by an increase of pressure translates to improved cell disintegration. However, this has a downside as well, meaning that the intracellular content of lytic enzymes is brought in contact with large amounts of suitable substrates. This might cause a drop in protein concentrations, in conjunction with a higher number of passes, as other studies relied on several passes - between 1 and 3. Considering we used much higher pressure and number of passes than the studies, it might point to a reason why with increasing pressure and increased enzyme concentration the protein yield drops.

The combination of piston homogenizer pressure and enzymatic treatment with yeast cell wall lysing enzymes proved to be very efficient in releasing yeast cell content. Separation of these two components of the yeast cell, yeast cell wall and intracellular proteins and peptides is important for further development of plant biostimulant.

Yeast cell wall acts as an elicitor of the plant innate immunity, due to its content of (1→3)-β-D-glucan (Sun et al., 2019) and chitin (Sun et al., 2018). Both (1→3)-β-D-glucan and chitin are active elicitors from the category of microbe-associated molecular patterns (MAMPs) (Boller & Felix, 2009). Till now, were registered as active ingredients for plant protection products the cell walls of the of *S. cerevisiae* strain LAS117, under common name cerevisane (EFSA, 2014) and lysate of the cell walls of *S. cerevisiae* strain DDSF 623), under common name ABE-IT 56. Yeast cell walls have a significant potential as low risk biopesticide, to control economic important plant diseases, such as downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator*) and grey mould (*Botrytis cinerea*) in grape (Angelini et al., 2019). Yeast intracellular proteins and peptides could be further converted into plant biostimulants by hydrolysis with proteases, to capitalize the existence of active ingredients acting on plants, such as glutamic acid (Lee et al., 2021), or glutathione (Ur Rehman et al., 2021).

The yeast extract itself proved to be effective in enhancing plant response to abiotic stress (Abdel Latef et al., 2019). Therefore, the potential agricultural applications of spent brewery yeast compensate its lack of attractivity to food industry.

CONCLUSIONS

The goal of this study was to devise an experimental plan and find a set of optimised values for the relevant parameters. ANOVA analysis revealed that in a high-pressure homogenisation setting, combined with an enzymatic pre-treatment, pressure and enzyme concentration seem to work synergically, to release a higher amount of protein from yeast cells. However, a more intense process does not seem to have a favourable effect on the yeast protein extraction, possibly due to interaction between the intracellular lytic enzymes and the protein content of these cells.

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