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Fodder yeast development optimisation using as main carbon source barley husks hydrolysed

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Abstract

Assessing the possibility of using the hydrolysed husks as carbon source for the production of fodder yeasts useful as supplements in animal nutrition was the aim of this work. The appropriate hydrolysis of barley husks lignocellulosic content can lead to high amounts of fermentable sugar useful for the growth of two different yeast strains (Candida utilis and Candida arboreea) which were chosen for their ability to utilize a wide variety of carbon sources and to conduct to high protein yield.

A particular interest was directed to the optimization of three different parameters: sugar content from hydrolysed barley husks, ammonium sulphate and magnesium sulphate contents, known as affecting the biomass development, using the response surface methodology (RSM).

Based on the proposed models, the optimum conditions for Candida utilis production were found to be at 18.81 g/L for sugar concentration, 8.71 g/L for ammonium sulphate and 0.69 g/L for magnesium sulphate. Under these conditions, the estimated biomass content was 6.69 g/L and protein content at 51.15% (w/w dry matter). For Candida arboreea strain the analysis revealed as optimum the following conditions: 18.42 g/L sugar concentration, 8.95 g/L ammonium sulphate and 0.66 g/L magnesium sulphate for biomass and protein contents of 6.74 g/L and 49.37% (w/w dry matter).

Keywords: Barley husk, *Candida utilis*, *Candida arborea*, Response Surface Methodology

Introduction

According to FAO statistics, barley (*Hordeum vulgare*) is the world's most important cereal after rice, maize and wheat. More than 123 million of tones were obtained in 2010 representing about 5% of the total cereals production across the world [1].
Barley is mainly used for animal feed or as a raw material in beer production. Its grains are structured on three main parts: the germ, the endosperm (comprising the aleurone and starchy endosperm) and the grain coverings. The last part may be divided into three fractions: the seed coat, the innermost layers surrounding the aleurone and the pericarp layers which are overlying the seed coat and which are covered by a multilayered tissue called "husk" [2].
Barley cleaning before use leads to solids waste mainly composed of husks, but also containing a minor proportion of grain fragments. According to Garrote et al. [3] burning of this material is not practical due to its high ash content, which results in mineral depositions in boilers. On the other hand, its low density makes the transport to disposal areas expensive. As consequence, various different other utilizations have been developed. Thus, Bledzki et al. [4] inspected the feasibility of utilizing barley husk and coconut shell as alternative fillers for soft wood fibre as reinforcement for composites material. They concluded that both these

materials are thermally stable at high temperatures and that barley husks showed better tensile strength than soft wood composites. Other studies [5-6] evaluated the possibility to use barley husks for ethanol production. Kim et al. [5] studied the effects of soaking the barley husks in aqueous ammonia as pretreatment for the production of ethanol and of additional xylanase on simultaneous saccharification and co-fermentation reaction of pretreated barley husks. Enzymatic saccharification was conducted to evaluate the obtained material's potential for bioconversion to fuel ethanol and/or for use as a ruminant (dairy and beef cattle) feed component with enhanced digestibility. The second study [6] was focused on the optimization of steam pretreatment of barley husk for high pentose and hexose recovery in the subsequent enzymatic hydrolysis step, as well as high ethanol yield, following simultaneous saccharification and fermentation. Several other studies [7-8] revealed that antioxidants derived from barley husks may be useful in the development of active packaging films for food preservation since the results confirmed that these antioxidants are able to slow down lipid hydrolysis and to increase the oxidative stability of food products. There are also researches indicating that barley husks can be successfully used for textile dye removal from aqueous effluents [9-11]. Knowing that husks, along with barley spent grains, are rich in various polysaccharides (hemicelluloses being the most important) a particular attention was accorded to their recovery. Several technologies have been developed to this purpose. They are based especially on different types of hydrolysis such as the enzymatic hydrolysis [12], dilute-acid hydrolysis [13] or autohydrolysis [3, 14] but they can use also the effect of microwave irradiation [15], the alkali extraction [16-17] or steam explosion and ultrafiltration [18]. Once recovered, these polysaccharides can be employed in food production since they influence the nutritive value and functional properties [19], in chemicals development or in ethanol obtaining process [20]. They can be also used as carbon source for yeasts growth such as *Debaryomyces hansenii* [13].

The aim of this work was to assess the possibility of using the hydrolysed husks as carbon source for the production of fodder yeasts useful as supplements in animal nutrition. Two yeast strains: *Candida utilis* and *Candida arboreea* were used to this purpose due to their ability to utilize a wide variety of carbon sources and to conduct at high yields of protein [21]. Our interest was also directed to the optimization of three different parameters known as affecting the biomass development. Sugar content from hydrolysed barley husks, ammonium sulphate and magnesium sulphate content influence on process evolution were followed. As consequence, the response surface methodology (RSM) was applied since it constitutes an effective statistical technique for optimizing complex processes which allows more efficient and easier arrangement and interpretation of experiments. In addition, it is less laborious and time-consuming than other approaches used to optimize a process [22].

A central composite design with three variables and three levels of variation was employed to obtain the best possible combination of the aforementioned parameters in order to achieve a maximum biomass production and high protein content.

Materials and methods

Chemicals

The reagents and solvents used were of analytical grade or comparable purity. They were obtained from Sigma Aldrich (Redox Lab Supplies Bucharest, Romania). Standard solutions were prepared by dissolving appropriate amounts of the commercially available pure products in double distilled water or adequate solvents.

Raw material

Barley husks are light yellow colour fibres and were collected from a local beer producer. Through their appropriate hydrolysis, the lignocellulosic biomass has been transformed into fermentable sugar (Table 1) and used as cultural substrate for growth of microorganisms.

An FT-IR Bruker Tensor 27 spectrophotometer set at 540 nm and 3,5-dinitrosalicylic acid reagent were used for the determination of the amount of reducing sugars from hydrolysed barley husks.

A stock solution of barley husks hydrolysed with a fermentable sugar concentration of 28 ± 1 g/L was prepared and then diluted to the desired concentrations according to the experimental conditions [23].

Table 1. The main fermentable sugar composition of hydrolysed barley husks

Constituent	Concentration, %	Constituent	Concentration, %
Glucose	54.5 ± 2	Xylose	38.3 ± 1
Arabinose	5.5 ± 0.5	Galactose	1.63 ± 0.2

Yeast strain and media

The yeasts used in this study were from the Collection of Microorganisms of SC ROMPAK SRL Paşcani. Yeasts cultures of *Candida arboreea* and *Candida utilis* were maintained on a solid yeast medium containing: D-glucose 20 g/L, Bacto peptone 10 g/L, yeast extract 5 g/L and agar 20 g/L. For the preparation of inoculum, *Candida* strains were transferred from agar slants into test tubes containing each 10 mL of sterile liquid yeast medium (D-glucose 20 g/L, Bacto peptone 20 g/L and yeast extract 10 g/L) and incubated at 30 °C for 24 h. 10 g/L of yeast inoculum was added in each experiment run.

The composition of the basal medium for control culture was: hydrolysed husks solution with 18 g/L sugar concentration and addition of $(NH_4)_2HPO_4$ 2 g/L, $FeSO_4$ 1 g/L, KCl 1 g/L, $ZnSO_4$ 1 g/L, $MnSO_4$ 1 g/L. In the basal medium, for yeast biomass cultivation in experimental conditions, the fermentable monosaccharides from hydrolysed husks solution varied between 16 and 20 g/L. $(NH_4)_2SO_4$ within a minimum/maximum of 7 and 9 g/L and $MgSO_4$ between 0.5 and 0.7 g/L were added. The variation of sugar concentration and mineral salts quantities was imposed by the experimental algorithm. The medium was sterilized at 120 °C for 15 min and after cooling to 30 °C it was centrifuged at 4000 rpm for 10 min. Clear supernatant was inoculated with yeast strains.

Experimental apparatus used in this stage were: trinocular microscope NOVEX, K Series, Model 85 340, 10x, 40x, 100x, with a vertical photo tube, hood with sterile air (laminar flow), "SPACE" PBI, 120/180, MEDIACLAVE 10 - Media sterilizer and Heraeus Megafuge 16 centrifuge, 4x400 mL polypropylene bio-bottle.

Culture conditions

Yeasts multiplications were performed in accordance with the experimental algorithm and semi-aerobic (thermostat) conditions at 38 °C for 48 h and an air flow of 0.02 L/h.

For batch processes 2.5 L of basal medium were introduced in a 5 L bioreactor tank equipped with steering system (adjustable speed 10-1000 rpm), heater system up to 100 °C, and air and ingredients dosing systems.

The pH of the medium was adjusted to 5.5 with $Ca(OH)_2$ with a concentration of 0.5 mol/L.

Experimental design

A central composite rotatable design (CCRD) was applied to select the independent variables and experiments for the optimization of biomass production using RSM analysis. The CCRD

considered the effects of three independent variables such as sugar content from hydrolysed barley husks, ammonium sulphate content and magnesium sulphate content with values taken within the ranges of 16 – 20 g/L, 7 – 9 g/L, 0.50 – 0.70 g/L respectively. Three levels for each independent variable (−1, 0, +1) were then chosen with coded values x_i obtained according to the following equation:

$$x_i = \frac{X_i - X_{i0}}{\Delta X_i} \quad (1)$$

where X_i, is the real value of the i^{th} independent variable, X_{i0} is its value in the central point of the interval and ΔX_i is the half of the difference between its upper and lower value.
Table 2 collects the coded and the real values for the three independent variables in the series of experiments from the CCRD.

Table 2. Code and level of independent variable chosen for the RSM test for *Candida utilis* or *Candida arboreea* multiplication

Variables	Symbol		Levels			ΔX
	Coded	Uncoded	-1	0	1	
			Actual values			
Sugar content g/L	x_1	X_1	16.00	18.00	20.00	2.00
Ammonium sulphate, g/L	x_2	X_2	7.00	8.00	9.00	1.00
Magnesium sulphate, g/L	x_3	X_3	0.50	0.60	0.70	0.10

Biomass content and protein percentage were chosen as the observed responses. They have been correlated with the coded values of the variables by means of the following general second-order polynomial equation:

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j \quad (2)$$

where Y is the observed response, A_0 is a constant coefficient, A_i, A_{ii} and A_{ij} are the coefficients for the linear, quadratic and interaction effects and X_i and X_j are the independent variables ($i \neq j$).

Three replicates of the central point were additionally made to estimate the experiments error. The response surfaces were generated with NemrodW v. 2000 program to find the best region for high biomass content and protein percentage. The validation of this model was confirmed by *ANOVA* analysis (XLSTAT-Pro 7.5 version).

Biomass content determination

Dry matter of yeast biomass was determined after washing the cells repeatedly with distilled water and drying biomass at 105 °C to a constant weight after centrifuging 10 mL samples at 4000 rpm for 15 min on Hettich Table Centrifuge.

Protein content determination

The content of total nitrogen from biomass (estimation of protein percentage with a correlation coefficient of 6.25) [24] was determined through the Kjeldahl method using a Hach - Digesdahl Digestion Apparatus and an Auto Analyzer, model 1030, Tecator, Hoganas by the standard procedure described in AOAC [25].

Results and discussion

Optimization of fodder yeasts development conditions by RSM

A total of 27 runs were used for optimizing individual parameters in the current CCRD, which was applied to the accumulation of biomass and total nitrogen for the two species of fodder yeast. The response values at different experimental combinations and equivalent predicted data from mathematical model created for each response variable are listed in Table 3.

Table 3. Experimental and predicted data for biomass and total nitrogen accumulation for the two strains of yeasts

Run	x_1	x_2	x_3	Biomass g/L				Protein content %, w/w dry matter			
				CU* Exp.**	CU Pred.**	CA* Exp.	CA Pred.	CU Exp.	CU Pred.	CA Exp.	CA Pred.
1	-1	-1	-1	4.400	4.065	4.210	3.969	33.330	31.005	30.650	29.100
2	-1	-1	0	4.550	4.594	4.420	4.451	33.730	34.556	31.550	32.320
3	-1	-1	1	4.660	4.605	4.500	4.342	34.820	34.768	32.740	31.784
4	-1	0	-1	5.000	5.010	4.870	4.893	38.190	38.419	35.810	36.080
5	-1	0	0	5.300	5.529	5.170	5.431	40.870	41.908	38.390	39.646
6	-1	0	1	5.450	5.530	5.320	5.376	41.960	42.059	39.480	39.457
7	-1	1	-1	5.300	5.489	5.170	5.254	40.870	41.860	38.390	38.587
8	-1	1	0	6.000	5.998	5.870	5.847	44.940	45.288	42.360	42.499
9	-1	1	1	6.150	5.989	5.880	5.848	46.530	45.378	42.760	42.656
10	0	-1	0	7.000	6.980	6.670	6.784	52.980	53.092	48.510	49.676
11	0	-1	0	6.050	6.980	5.920	6.784	46.830	53.092	44.150	49.676
12	0	-1	0	7.100	6.980	6.970	6.784	54.760	53.092	51.690	49.676
13	0	0	0	7.450	7.172	7.300	7.056	57.440	54.764	54.170	51.801
14	0	0	0	7.350	7.172	7.270	7.056	55.650	54.764	52.880	51.801
15	0	0	0	7.550	7.172	7.390	7.056	57.140	54.764	53.770	51.801
16	0	1	0	6.850	6.899	6.720	6.763	51.790	52.464	48.910	49.453
17	0	1	0	6.950	6.899	6.820	6.763	52.580	52.464	49.600	49.453
18	0	1	0	6.850	6.899	6.750	6.763	51.790	52.464	49.110	49.453
19	1	-1	1	6.900	6.714	6.560	6.451	52.180	50.898	47.720	47.174
20	1	-1	0	7.300	6.808	6.960	6.522	54.960	51.285	50.600	47.298
21	1	-1	-1	6.150	6.384	5.880	6.001	46.530	48.333	42.760	43.667
22	1	0	1	5.880	6.154	5.750	6.069	44.440	46.829	41.870	44.445
23	1	0	0	6.080	6.258	6.010	6.085	45.730	47.277	43.450	44.223
24	1	0	-1	5.780	5.844	5.450	5.509	43.750	44.386	39.680	40.245
25	1	1	1	5.080	5.128	5.200	5.124	38.790	38.788	38.190	37.243
26	1	1	0	5.200	5.242	4.990	5.084	39.380	39.297	36.310	36.674
27	1	1	-1	5.000	4.838	4.500	4.454	37.800	36.467	32.740	32.350

* CU – *Candida utilis*; CA – *Candida arboreea*; ** Exp. – experimental; Pred. – predicted

The obtained equations allowed the prediction of the effects of the three factors on the biomass production and protein content. The independent response surface plots and their respective contour plots are shown in Figures 1, 2, 3 and 4. Two variables within the experimental rang were depicted in 3-D surface plots, while the two other variables were kept

constant at zero level. The shapes of the contour plots, circular or elliptical indicated whether the mutual interactions between the variables were significant or not.
All three factors, namely sugar concentration of hydrolysed barley husks (X1), ammonium sulphate (X2) and magnesium sulphate (X3) had a positive impact on the fodder yeast production.

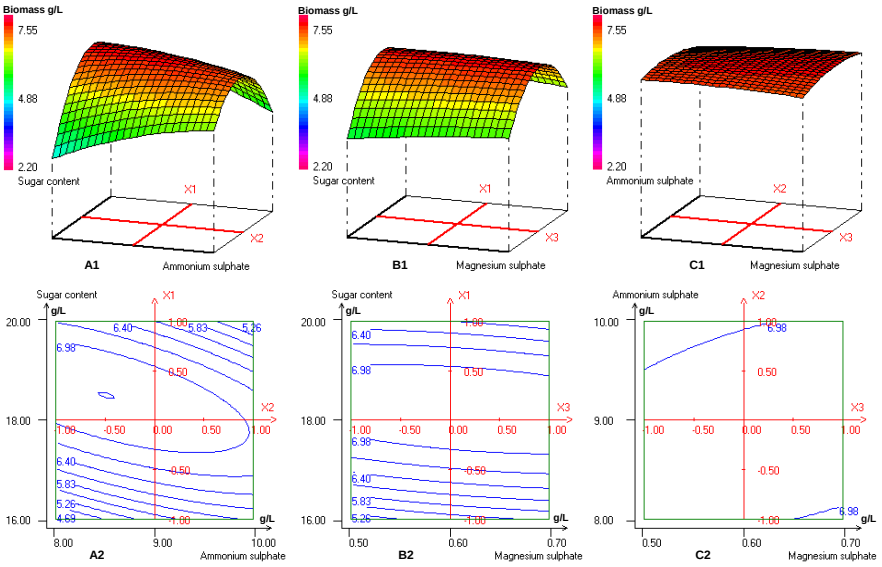

Figure 1. Response surface plots (1) and contour plots (2) for the effects of (A) sugar content and ammonium sulphate; (B) sugar concentration and magnesium sulphate; (C) ammonium sulphate and magnesium sulphate on the biomass for *Candida utilis*

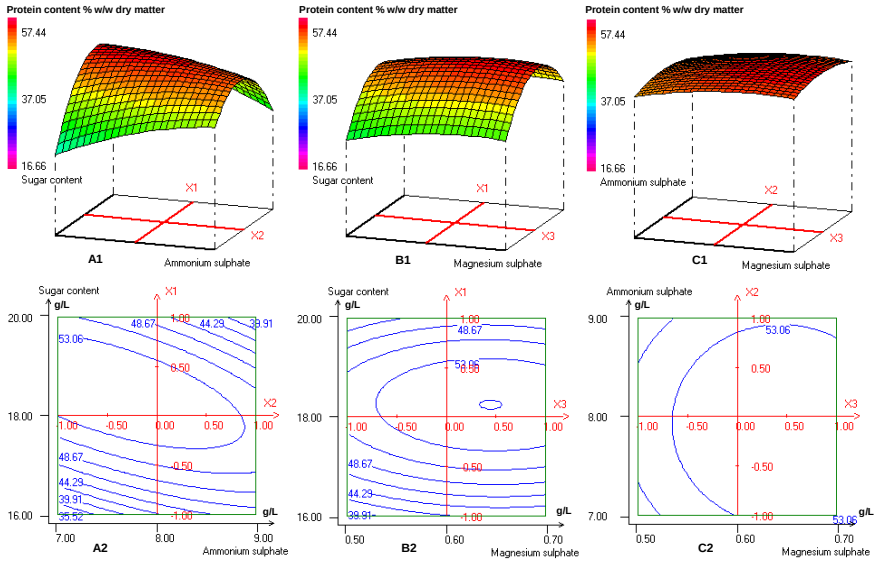

Figure 2. Response surface plots (1) and contour plots (2) for the effects of (A) sugar content and ammonium sulphate; (B) sugar concentration and magnesium sulphate; (C) ammonium sulphate and magnesium sulphate on the protein content for *Candida utilis*

By applying the multiple regression analysis on the experimental data of the *Candida utilis* yeast multiplication process, the response variable and the test variable were related through the following second-order polynomial equations:

Biomass content equation:

$$Y = 7.172 + 0.364 \cdot x_1 - 0.041 \cdot x_2 + 0.208 \cdot x_3 - 0.743 \cdot x_1 \cdot x_2 - 0.053 \cdot x_1 \cdot x_3 + \\ + 0.010 \cdot x_2 \cdot x_3 - 1.278 \cdot x_1^2 - 0.233 \cdot x_2^2 - 0.259 \cdot x_3^2 \quad (3)$$

Protein content equation:

$$Y = 54.764 + 2.684 \cdot x_1 - 0.314 \cdot x_2 + 1.521 \cdot x_3 - 5.680 \cdot x_1 \cdot x_2 - 0.299 \cdot x_1 \cdot x_3 + \\ + 0.061 \cdot x_2 \cdot x_3 - 10.172 \cdot x_1^2 - 1.986 \cdot x_2^2 - 1.669 \cdot x_3^2 \quad (4)$$

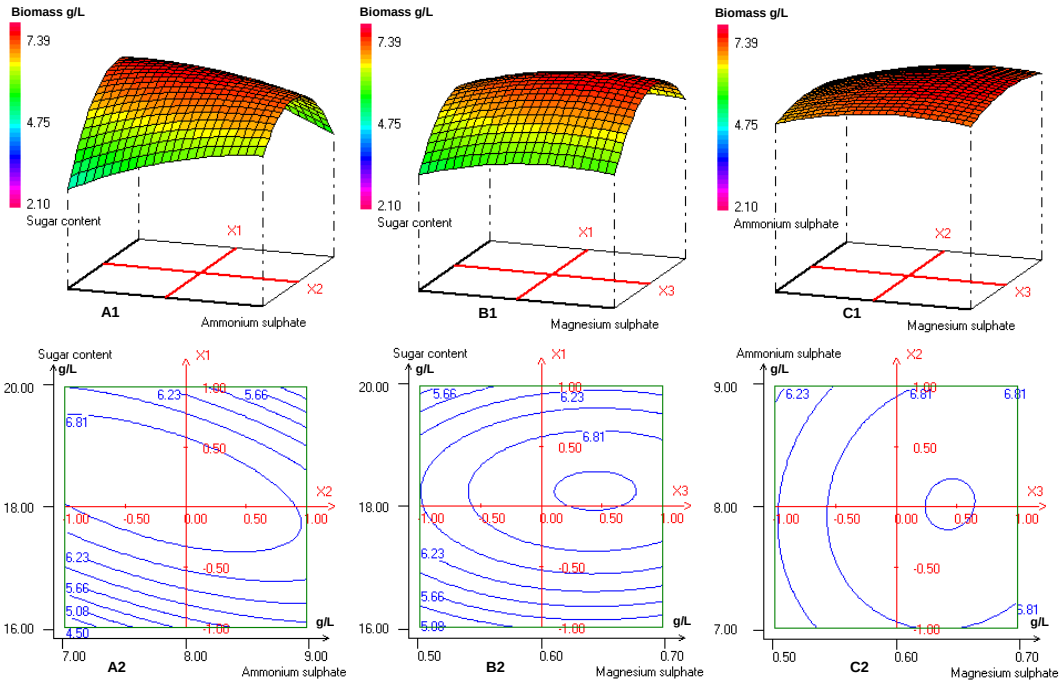

Figure 3. Response surface plots (1) and contour plots (2) for the effects of (A) sugar content and ammonium sulphate; (B) sugar concentration and magnesium sulphate; (C) ammonium sulphate and magnesium sulphate on the biomass for *Candida arboreea*

The data analysis for the biomass content indicated that the coefficient of determination (R^2) of the predicted model was 0.925 and that the adjusted determination coefficient (Adj. R^2) was 0.885, suggesting a good fit; the predicted model seemed to reasonably represent the observed values. The significance of the response equation coefficients was $p = 0.0245$ for A_1 and $p < 0.01$ for A_{11} and A_{12} indicating that the sugar concentration influence biomass production. The p values are used to check the significance of each coefficient, which in turn may indicate the pattern of the interaction between the variables.

For protein content, data analysis for the quadratic regression model showed that the values of the determination coefficient (R^2) and the adjusted determination coefficient (Adj. R^2) were 0.934 and 0.899, respectively, which suggested an important degree of correlation between the observed and the predicted values. The mathematical analyses showed that the sugar concentration influences (significance, $p = 0.0188$ for A_1 and $p < 0.01$ for A_{11}, A_{12}) the protein amount determinate in the experiments.

The RSM was applied also in the case of *Candida arboreea* multiplication process. The obtained results revealed that the response variable and the test variable were related through the following second-order polynomial equations:

Biomass content equation:

$$Y = 7.056 + 0.327 \cdot x_1 - 0.011 \cdot x_2 + 0.261 \cdot x_3 - 0.708 \cdot x_1 \cdot x_2 + 0.019 \cdot x_1 \cdot x_3 + \\ + 0.055 \cdot x_2 \cdot x_3 - 1.298 \cdot x_1^2 - 0.282 \cdot x_2^2 - 0.296 \cdot x_3^2 \quad (5)$$

Protein content equation:

$$Y = 51.801 + 2.288 \cdot x_1 - 0.111 \cdot x_2 + 1.894 \cdot x_3 - 5.201 \cdot x_1 \cdot x_2 + 0.206 \cdot x_1 \cdot x_3 + \\ + 0.346 \cdot x_2 \cdot x_3 - 9.867 \cdot x_1^2 - 2.237 \cdot x_2^2 - 1.878 \cdot x_3^2 \quad (6)$$

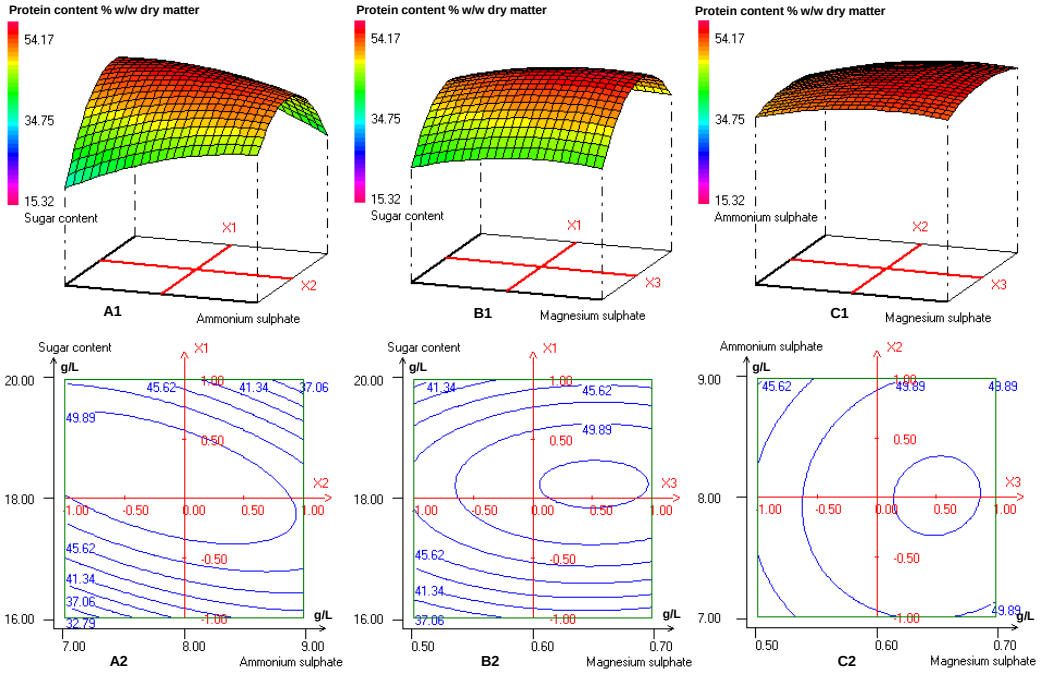

Figure 4. Response surface plots (1) and contour plots (2) for the effects of (A) sugar concentration and ammonium sulphate; (B) sugar concentration and magnesium sulphate; (C) ammonium sulphate and magnesium sulphate on the protein content for *Candida arboreea*

The data analysis for the biomass content for *Candida arboreea* indicated that for predicted model R^2 was 0.938 and Adj. R^2 was 0.905. The significance of the response equation coefficients was $p = 0.0275$ for A_1 and $p < 0.01$ for A_{11} and A_{12} indicating an influence of sugar concentration over the biomass content.

For protein content, the data analysis for the quadratic regression model showed that the values of the determination coefficient (R^2) and the adjusted determination coefficient (Adj. R^2) were 0.945 and 0.915. A good fit between the experimental and the predicted data was observed and their analysis showed that the sugar concentration has the influence on the protein amount determinate in the experiments (significance, $p = 0.0253$ for A_1 and $p < 0.01$ for A_{11}, A_{12}).

An influence was recorded even for the ammonium sulphate but the level of significance was small for both response functions.

Optimization of the procedure

The real values of the independent variables for the optimum results were calculated targeting the maximum values for all the response variables. The obtained values are presented in Table 4.

Table 4. Values for optimum amounts of sugar content, ammonium and magnesium sulphate

Response variable, maximum obtained values (Y)	Sugar concentration, g/L (X_1)	Ammonium sulphate, g/L (X_2)	Magnesium sulphate, g/L (X_3)
Candida utilis			
Biomass 6.69 g/L Protein content 51.15 %*	18.81	8.71	0.69
Candida arboreea			
Biomass 6.74 g/L Protein content 49.37 %*	18.42	8.95	0.66

* %, w/w dry matter

Models validation

In order to validate the adequacy of the model equations, 4 replicates of the experiment were carried out under the above mentioned optimal conditions. The standard *F-Test* showed minor differences, 6.69 g/L ± 0.91% for biomass content and 51.15% ± 0.76% for protein content for *Candida utilis* and 6.74 g/L ± 0.67% for biomass content and 49.37% ± 0.32% for protein content for *Candida arboreea* between response variable presented in Table 4 and the experimental results.

Compared to the values obtained for control yeast culture (3 replicates) a significant increase was observed for biomass and protein contents and the differences were in limit of 23.76 ± 0.8% and 26.26 ± 1.8% for *Candida utilis* respectively 25.7 ± 0.9% and 27.46 ± 2.22% for *Candida arboreea*.

Conclusion

The research demonstrates that hydrolysed barley husks can be successfully employed as carbon source for fodder yeast production with similar results for biomass and protein contents as those reported when substrates such as pineapple juice production wastes [26-27] or rice straw hydrolysed [28] are used.

The employed experimental design and approach enabled to establish the polynomial functions that describe the effects of sugar concentration, ammonium sulphate and magnesium sulphate on fodder yeast production.

The proposed polynomial regression models described the experimental results satisfactorily. Based on the proposed models, the optimum conditions for *Candida utilis* production were found to be at 18.81 g/L for sugar concentration, 8.71 g/L for ammonium sulphate and 0.69 g/L for magnesium sulphate. Under these conditions, the estimated biomass content was 6.69 g/L and protein content at 51.15%. Similar results were obtained for the production of fodder yeast when using *Candida arboreea* strain. The analysis showed that the optimum conditions were: 18.81 g/L sugar concentration, 8.95 g/L ammonium sulphate and 0.66 g/L magnesium sulphate. In this case, the biomass content was 6.74 g/L with 49.37% protein. Even that the *Candida utilis* scores better results in protein content in the same experimental conditions than *Candida arboreea*, the differences were minor being with 0.7% less in biomass and respectively 3.47% higher in protein content for the first yeast.

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