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NEW POSSIBILITIES OF FODDER YEAST PRODUCTION

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Key words: *wastewater, pulp and paper, beet-pulp, hydrolysis, fodder yeast, Candida utilis*

INTRODUCTION

Pulp and paper industry is considered one of the major consumers of energy and natural resources. The process requires up to 60 m^3 of freshwater per ton of produced paper [Thompson 2001]. This process is also a significant contributor to pollutant discharges into the environment [Plant 2014, Raud 2012]. Suspended solids, color compounds, heavy-metals, organic and inorganic substances, phenol, chloro-organics, cyanide, sulphides, and other soluble substances [Achoka 2002, Simonic 2012] can be detected especially in the wastewater released at the end of the manufacturing process. Due to its heterogeneous composition [Maximova 2007], the industrial wastewater can cause slime growth, thermal impacts, color problems, and a loss of environment's aesthetic beauty. The increased amount of toxic substances in the water, can lead to plankton and fish death, and can affect the terrestrial ecosystem. Consequently, new approaches in wastewater treatment technology were developed in order to comply with the more stringent environmental regulations on the quality of effluent entering recipient waters. The wastewater can be submitted to various decontamination steps by using methods such as adsorption, advanced oxidation, membrane filtration [Buyukkamaci 2010], coagulation and flocculation [Ahmad 2008], solar photo-catalysis [Amat 2005], electro coagulation [Katal 2011], catalysed ozonation [Fontanier 2006], or solar photo-Fenton processes [Xu 2007].

Another very important industrial activity known as leading to serious environmental issues is represented by the sugar industry. High quantities of wastes both liquids and solids are also obtained the sugar beet pulp being the most important one. Different researches have shown that this by-product is particularly suitable as fodder in cattle-raising sector [Pereira 2004], for the production of biofuels [Kato 2000, Spagnuolo 1999, Zheng 2011], pectin extract [Favela-Tores 2006, Siew 2008], biodegradable plastics [Liu 2005], antioxidants [Sakac 2011, Sakac 2009, Mohdaly 2010] etc. Due to its high content of hemicelluloses and celluloses, the beet-pulp can be exploited also as source of reducing sugars. A method based on acid hydrolysis was developed to this purpose and its uses were described in ones of our previous published papers [Simion 2012].

The study of pulp and paper wastewaters and beet-pulp hydrolysed composition revealed that both are especially rich in reducing sugars, constituents that can be successfully employed in different other industrial sectors such as fodder yeast production. *Candida utilis* is a popular microorganism which presents high respiratory activity, high protein content (up 50% of the dry weight) [Lee 2001], good amino acids profile and ability to utilize a wide range of substrates [Ghoul 1991]. Furthermore, the predominantly aerobic metabolism of *Candida utilis* and active participation of the pentose phosphate pathway for sugar metabolism predisposes this yeast to carbon balance in favor of biomass production when compared to other yeasts such as *Saccharomyces cerevisiae*, which is glucose sensitive and largely fermentative [Pinheiro 2014]. *Candida utilis* strains have been used for production of biotin, ethanol or lactate [Hong 2006, Ikushima 2009, Tamakawa 2010]. This yeast can be also incorporated in animal diet as supplement in order to insure the adequate nutritive intake of vitamins and minerals.

As consequence, we have focused our research on the investigation of the optimal conditions able to conduct to important contents of biomass and protein when using wastewaters from pulp and paper industry and beet-pulp hydrolysed as carbon source for the development of *Candida utilis* strains. Three different factors (total sugar, nitrogen and phosphate contents) and three levels of variations were used in an experimental program of 27 runs with 3 replicates in the central point. The Response Surface Methodology (RSM) was applied to identify the influence of the aforementioned parameters on the production of fodder yeast.

MATERIALS AND METHODS

Reagents

Analytical grade reagents and solvents used were obtained from Sigma Aldrich (Redox Lab Supplies Bucharest, Romania). Standard solutions were prepared by dissolving appropriate amounts of pure products in demineralized water or adequate solvents.

Wastewaters from pulp and paper industry

Pulp and paper wastewaters used in this research as main carbon source for yeast development due to their high monosaccharides content are the result of sulphite pulping process. Their physicochemical characterisation is shown in Table 1.

The monosaccharides existing in these wastewaters are: 17% galactose, 10% glucose, 44% mannose, 7% xylose, 4% arabinose, 21% glucuronic acid.

The mineral content (% w/w ash) is: 1.75% calcium, 0.30% manganese, 0.08% magnesium, 7.25% sodium, 0.04% zinc, 0.02% iron.

Beet pulp hydrolysed

The hydrolysed was obtained by treating the spent beet-pulp noodles with sulphuric acid in two hydrolysis stages [Simion 2012]. Its average chemical composition is presented in Table 2.

In terms of monosaccharides content, glucose (22.9%), galacturonic acid (15.1%), galactose (4.8%), xylose (1.2%), mannose (0.9%), rhamnose (0.8%) are the most important ones.

Yeast multiplication

The yeast culture of *C. Utilis* provided by Centraalbureau voor Schimmelcultures, Delft, The Netherlands was maintained on a solid yeast medium containing: D-glucose 20 g/L, Bactopeptone 10 g/L, yeast extract 5 g/L and agar 20 g/L. The culture was transferred from agar slants into test tubes containing each 10 mL of sterile liquid yeast medium (D-glucose 20 g/L, Bactopeptone 20 g/L and yeast extract 10 g/L) and incubated in a thermostat at 30 °C for 24 h in order to prepare the inoculum (10 g/L).

For control culture the basal medium composition was: 50 % pulp and paper wastewaters and 50% sugar-beet hydrolysed (after dilution of each with demineralized water to 18-26 g/L fermentable monosaccharides, variation imposed by experimental algorithm) with addition of $MgSO_4$ 1 g/L, $ZnSO_4$ 1 g/L, $MnSO_4$ 1 g/L, $FeSO_4$ 0.8 g/L and KCl 1 g/L.

For yeast biomass cultivation in experimental conditions up to 1100 mg/L nitrogen and 420 mg/L phosphorous from $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ were added in the basal medium in quantities established by the experimental algorithm.

The medium was sterilized at 120 °C for 15 min, cooled to 30 °C and centrifuged at 4000 rpm for 10 min. The recovered clear supernatant was used as the basal medium.

The medium pH was adjusted to a value of 5.5 with a 5 M $Ca(OH)_2$ solution.

2.5 L of basal medium were introduced in a 5 L bioreactor tank and used for the batch processes at 38 °C for 48 h. Semi-aerobic conditions were insured by an air flow of 0.02 L/h.

Samples of 7.5 cm^3 were centrifuged, washed with distilled water, dried at 105 °C until constant weight and analyzed in triplicate for biomass content, total nitrogen (expressed as protein percentage) and residual sugar content.

Experimental apparatus used in this stage were: a trinocular microscope NOVEX, KSeries, Model85 340,10x, 40x, 100x, with a vertical phototube, a hood with sterile air (laminar flow), "SPACE" PBI, 120/180, a 5 L capacity bioreactor with steering system with adjustable speed 10-1000 rpm, heater system up to 100 °C, air and ingredients dosing systems.

A Hettich Table Centrifuge EBA III, 4x15 g load, adjustable speed 800-6000 rpm and a Kern MLB 50-3 Moisture Balance were also employed.

Biomass content determination

Dry matter of yeast biomass was determined after washing the cells 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.

Table 1. Characteristics of wastewaters from technological process of de-crusting of resinous wood with $NaHSO_3$

Characteristic	Value
Fibres suspensions, g/L	0.055
Fermentable monosaccharides g/L	59 (± 2%)
Phurphurol, mg/L	0.1-0.15
SO_2 organically bound, g/L	2-3
SO_2 free, g/L	0.5-1
Fix residue at 100 ± 5 °C, % w/w	58.50%
Total organic compounds TOC (% w/w fix residue at 100 ± 5 °C)	70%
Chemical oxygen demand COD, mg/L	85000
Biochemical oxygen demand at 5 days BOD_5, mg/L	7250
Ash at 800 ± 5 °C, % w/w	3.85
Density, kg/m^3	1060
pH	3.5-4.0

Table 2. Chemical composition of sugar beet pulp hydrolysed

Characteristic	Value
Dry matter, % w/w	17.42
Fermentable monosaccharides g/L	88 (± 3%)
Phurphurol, mg/L	0.21
Ash at 800 ± 5 °C, % w/w	2.66
Density, kg/m^3	1048
pH	4.0-5.0

Protein and total nitrogen content determination

The content of total nitrogen from biomass (and the estimation of protein percentage) was determined through the Kjeldahl AOAC method using a Hach - Digesdahl Digestion Apparatus and an Auto Analyzer, model 1030, Tecator, Hoganas. For estimating the protein content a correlation coefficient of 6.25 was used.

Residual sugar content determination

An HACH DR/2000 spectrophotometer set at 540 nm and 3,5-Dinitrosalicylic acid (DNS) reagent were used for the determination of the amount of reducing sugars in both of the solutions obtained.

Ash content determination

The ash content was determinate at 575 ± 25 °C, 24 h with a Caloris L 1003 Muffle Furnace according to a AOAC standard procedure.

Experimental design

The independent variables were coded (Table 3) by transforming each studied real value into coordinates inside a scale with dimensionless values, which are proportional to their location in the experimental space. The correspondence between the coded and the uncoded values is expressed by the equation 1

$$x_i = \left(X_i - X_{i0}\right) / \Delta X_i \qquad (1)$$

where x_i is the coded value of an independent variable; X_i is the real value of an independent variable; X_{i0} is the average between the maximum and the minimum values of the independent variable; ΔX_i is the step change value of an independent variable.

The correlation between the independent variables for each response function (biomass, protein and ash contents) was realised via a quadratic polynomial model. The general equation used for predicting the optimal point for all the studied parameters was:

$$_nY = A_0 + \sum_{i=1}^{3} A_i \cdot {}_nX_i + {}_n\sum_{i=1}^{3} A_{ii} \cdot {}_nX_i^2 + {}_n\sum_{i=1}^{2}\sum_{j=i+1}^{3} A_{ij} \cdot {}_nX_i \cdot {}_nX_j \qquad (2)$$

where the values of n are between 1 and 3, $_nY$ is the response function ($_1Y$ biomass gain, $_2Y$ protein content, $_3Y$ residual sugar content), $_nA_0$, $_nA_i$, $_nA_{ii}$, $_nA_{ij}$ are the regression coefficients of variables for the intercept, linear, quadratic and interaction terms, X_i and X_j are the independent variables ($i \neq j$).

Analyses of the experimental design and data were carried out using the NemrodW v. 2000 software.

Statistical analysis

For characterization of the samples, at least five replicates measurements were performed, unless otherwise specified.

The results were analyzed by using analysis of variance (ANOVA) (XLSTAT-Pro 7.5 version), and the t-Test. Results with a corresponding probability value of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSIONS

Independent variables effects on protein, biomass gain and residual sugar content

In order to fit mathematical models to the experimental data aiming at an optimal region for the studied responses a regression analysis (Table 4) was carried out.

Involving multiple regression analysis on the experimental data of yeast multiplication process, the response variable and the test variable were related through three quadratic polynomial equations.

For biomass gain (equation 3), the ANOVA test showed that the values of the determination coefficient (R^2) and the adjusted determination coefficient (Adj. R^2) were 0.977 and 0.965, respectively suggesting a high degree of correlation between the observed and the predicted values.

When applied for protein content, the ANOVA test indicated a coefficient of determination (R^2) of 0.930 and an adjusted determination coefficient (Adj. R^2) of 0.892, suggesting that the predicted model (equation 4) seemed to reasonably represent the observed values.

Table 3. Code and level of independent variable chosen for the RSM test for fodder yeasts multiplication

Variables	Symbol		Levels			Step change value ΔX
	Coded	Uncoded	-1	0	1	
			Actual values			
Total sugar content, g/L	x_1	X_1	18	22	26	4
Nitrogen content, mg/L	x_2	X_2	700	900	1100	200
Phosphate content, mg/L	x_3	X_3	340	380	420	40

Table 4. RSM test for fodder yeast multiplication and the observed and predicted values for the biomass, protein contents and residual sugar

Run	Total sugar content, g/L	Nitrogen content, mg/L	Phosphate content, mg/L	Biomass gain, g/L		Protein content, % w/w		Residual sugar content, g/L	
				Obs.*	Pred.**	Obs.	Pred.	Obs.	Pred.
1	18	700	340	3.80	3.547	29.22	26.199	1.71	1.624
2	18	700	380	4.05	4.050	29.90	28.997	1.23	1.304
3	18	700	420	4.07	4.186	30.16	28.849	1.24	1.133
4	18	900	340	4.42	4.608	-	34.659	1.01	1.141
5	18	900	380	4.93	5.016	37.49	38.070	0.94	0.915
6	18	900	420	5.10	5.057	37.82	38.536	0.89	0.837
7	18	1100	340	4.79	4.855	32.50	34.679	0.94	0.962
8	18	1100	380	5.18	5.168	39.55	38.705	0.86	0.829
9	18	1100	420	5.26	5.114	40.02	39.785	0.77	0.845
10	22	700	340	4.70	4.969	34.54	36.999	1.94	1.841
11	22	700	380	5.48	5.386	35.01	38.698	1.36	1.579
12	22	700	420	5.49	5.436	35.17	37.452	1.34	1.467
13	22	900	340	6.41	6.305	50.23	48.447	1.24	1.199
14	22	900	380	-	6.305	-	48.447	1.23	1.199
15	22	900	420	6.34	6.305	49.90	48.447	1.25	1.199
16	22	1100	340	5.99	6.271	48.15	49.740	1.19	1.197
17	22	1100	380	6.47	6.410	51.35	49.758	1.18	1.122
18	22	1100	420	6.37	6.183	51.07	46.831	1.27	1.197
19	26	700	340	5.47	5.486	36.72	35.449	3.14	3.080
20	26	700	380	5.45	5.521	36.87	37.794	3.17	3.134
21	26	700	420	5.26	5.190	40.04	37.193	3.37	3.338
22	26	900	340	6.32	6.263	48.62	46.489	2.85	2.803
23	26	900	380	6.39	6.393	48.00	48.219	2.77	2.764
24	26	900	420	6.18	6.157	45.44	47.004	2.75	2.874
25	26	1100	340	6.34	6.227	48.73	49.089	2.77	2.829
26	26	1100	380	6.39	6.452	-	50.206	2.71	2.696
27	26	1100	420	6.30	6.311	47.71	48.376	2.70	2.713

*Obs. – observed; **Pred. – predicted

$$ {}_1y = 6.305 + 0.686{}_1x_1 + 0.512\cdot{}_1x_2 + 0.139\cdot{}_1x_3 - 0.047\cdot{}_1x_1\cdot{}_1x_2 - 0.086\cdot{}_1x_1\cdot{}_1x_3 - 0.095\cdot{}_1x_2\cdot{}_1x_3 - $$
$$ - 0.600\cdot{}_1x_1^2 - 0.407\cdot{}_1x_2^2 - 0.183\cdot{}_1x_3^2 \quad (3) $$

$$ {}_2y = 48.447 + 5.074\cdot{}_2x_1 + 5.530\cdot{}_2x_2 + 0.841\cdot{}_2x_3 + 0.676\cdot{}_2x_1\cdot{}_2x_2 - 1.098\cdot{}_2x_1\cdot{}_2x_3 + 0.614\cdot{}_2x_2\cdot{}_2x_3 - $$
$$ - 5.303\cdot{}_2x_1^2 - 4.219\cdot{}_2x_2^2 - 1.473\cdot{}_2x_3^2 \quad (4) $$

$$ {}_3y = 1.199 + 0.924\cdot{}_3x_1 + 0.228\cdot{}_3x_2 - 0.094\cdot{}_3x_3 + 0.009\cdot{}_3x_1\cdot{}_3x_2 + 0.058\cdot{}_3x_1\cdot{}_3x_3 + 0.093\cdot{}_3x_2\cdot{}_3x_3 $$
$$ + 0.640\cdot{}_3x_1^2 + 0.152\cdot{}_3x_2^2 + 0.075\cdot{}_3x_3^2 \quad (5) $$

For residual sugar content (equation 5) the ANOVA test indicates that R^2 was 0.991 and Adj. R^2 was 0.987. The significance of the response equation coefficients indicates interactions between all three variables. The same influence of the sugar and nitrogen content in the basal medium was recorded over the residual sugars, but their amount could be influenced directly by the phosphorous content and indirectly by the sugar-phosphorus interaction.

The significance (Table 5) of the response equation coefficients indicate the same influence over the protein content of sugar and nitrogen concentration as in the case of biomass accumulation.

Testing of optimal process conditions

Plotting the response versus the experimental variables the relationship between them were illustrated graphically. The 3D response surfaces were formed by contour plots that represent curves of constant response on a two-variable plain. The graphical representations were created by setting the experimental factors at their intermediate levels. The

obtained plots (Figures 1, 2 and 3) were used in studying the effects of the variation of studied parameters in the established domain and, therefore, in determining the optimal experimental conditions.
Aiming the response variables at their maximum values for biomass and protein and at minimum for the residual sugar content the real values of the independent variables for the optimum results were calculated. The obtain values are given in Table 6.

In order to validate the mathematical models adequacy, 5 replicates of the experiment were carried out under the above mentioned optimal conditions. Comparing the values between the response variable presented in Table 6 and the experimental results for these replicates, only minor differences were observed (6.46 g/L ± 1.7% for biomass content, 50.50% ± 0.20% for protein content and 1.88 g/L ± 4.9% for residual sugar content).

Table 5. Significance of coefficients in the response equations

Coefficient	Significance, p %		
	Equation 3	Equation 4	Equation 5
A_0	< 0.01 ***	< 0.01 ***	< 0.01 ***
A_1	< 0.01 ***	< 0.01 ***	< 0.01 ***
A_2	< 0.01 ***	< 0.01 ***	< 0.01 ***
A_3	0.249 **	19.0	0.154 **
A_{11}	< 0.01 ***	0.0105 ***	< 0.01 ***
A_{22}	< 0.01 ***	0.0851 ***	0.200 **
A_{33}	1.06 *	16.3	8.1
A_{12}	31.6	36.0	75.0
A_{13}	7.1	13.9	5.5
A_{23}	4.81 *	40.6	0.456 **

Table 6. Desirability

Maximum coordinates			
Variable	Coded value	Factor	Real value
X_1	0.469925	Sugar content, g/L	24
X_2	0.691295	Nitrogen content, mg/L	1038
X_3	0.993292	Phosphorus content, mg/L	420
Maximum characteristics			
Response function			Value
$_1Y$	Biomass gain, g/L		6.49
$_2Y$	Protein content, % w/w		50.98
$_3Y$	Residual sugar, g/L		1.76

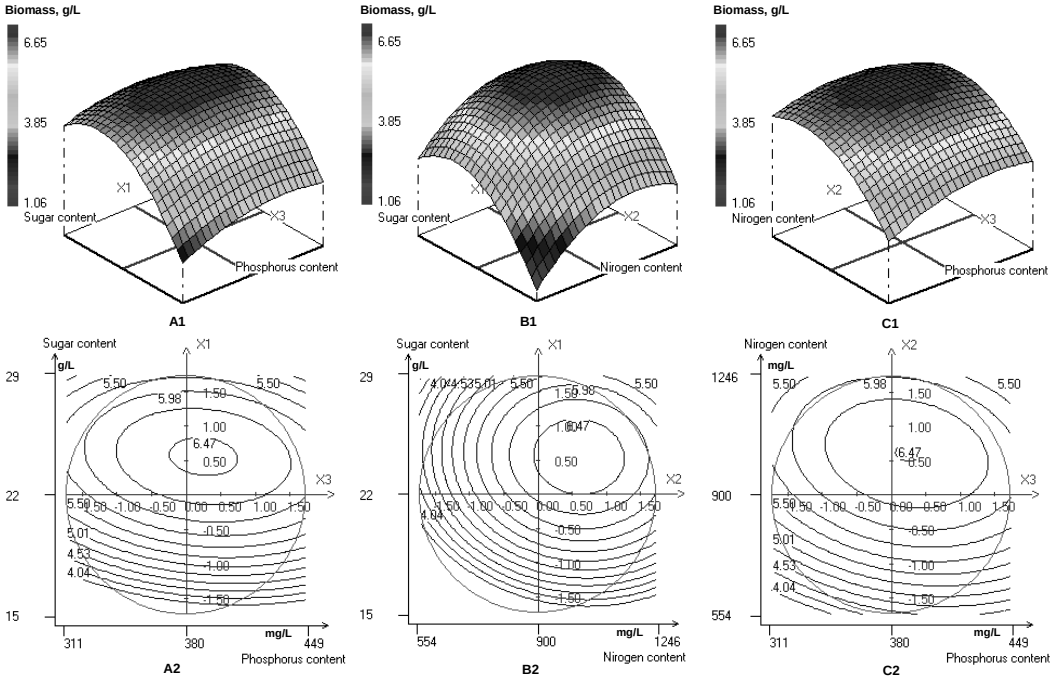

Fig. 1. Response surface plots (1) and contour plots (2) for the effects of (A) sugar content and nitrogen content; (B) sugar concentration and phosphorous content; (C) nitrogen and phosphorous content on the biomass gain

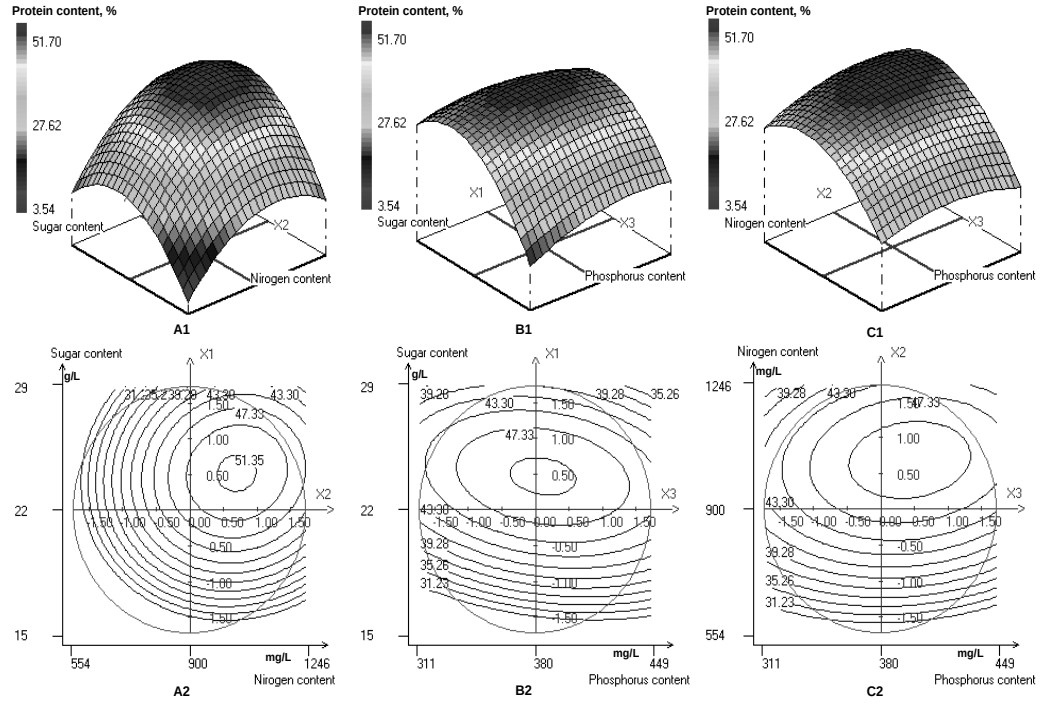

Fig. 2. Response surface plots (1) and contour plots (2) for the effects of (A) sugar content and nitrogen content; (B) sugar concentration and phosphorous content; (C) nitrogen and phosphorous content on the protein content

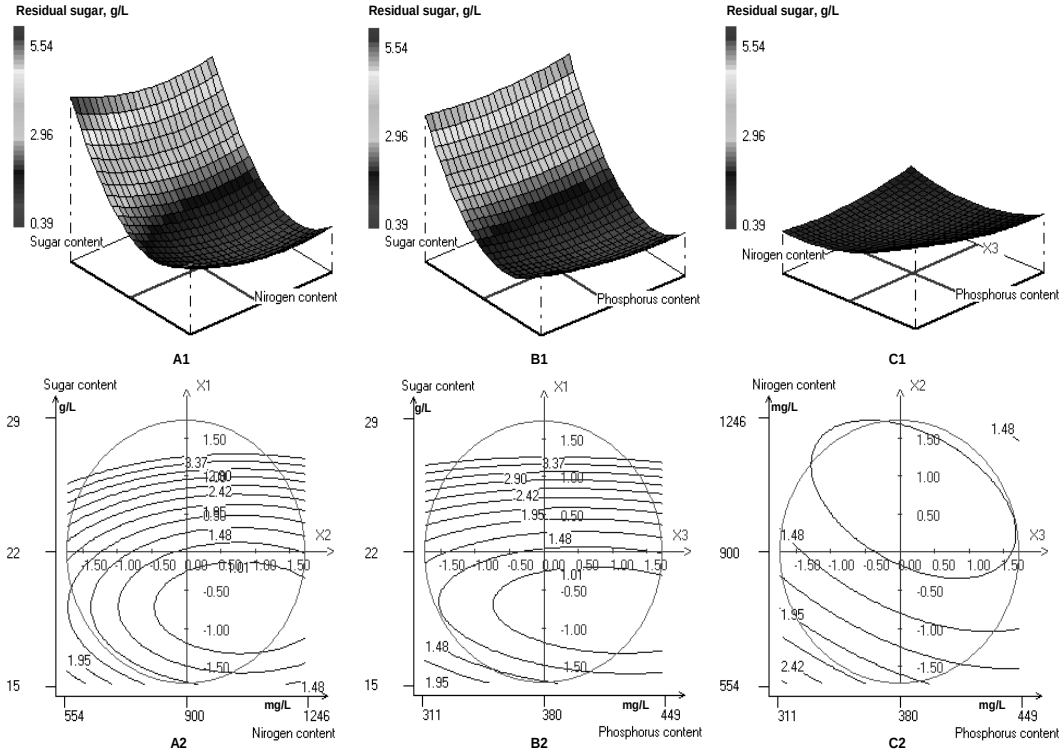

Fig. 3. Response surface plots (1) and contour plots (2) for the effects of (A) sugar content and nitrogen content; (B) sugar concentration and phosphorous content; (C) nitrogen and phosphorous content on residual sugar

CONCLUSIONS

This paper aimed to capitalize pulp and paper wastewaters and beet-pulp hydrolysed as main carbon source and integrate them in fodder yeast multiplication and development process. This product represents a valuable nutritive supplement rich in vitamins and minerals which can successfully complete the animals diet.

A mix of wastewaters from pulp and paper industry (59 ± 2% g/L fermentable sugars) and beet-pulp hydrolysed (88 ± 3% g/L fermentable sugars) was employed as primary carbon source on growth mediafor the development of an inoculum of *Candida utilis*.

The initial concentrations of sugar, nitrogen and phosphorous were optimised by applying the Response Surface Methodology. The RSM used to this purpose offered the following formulation for the culture media in batch system: 24 g/L fermentable monosaccharides, $MgSO_4$ 1 g/L, $ZnSO_4$1.0 g/L, $MnSO_4$1.0 g/L, $FeSO_4$ 0.8 g/L, KCl 1 g/L and up to 1038 mg/L nitrogen and 420 mg/L phosphorous from $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$. In these conditions, the biomass gain was of 6.49 g/L and the protein content reached a value of 50.98% while the amount of the residual sugar decreased at 1.76 g/L.

5 separated experiments were realized by applying these conditions and only minor differences were observed, 6.46 g/L ± 1.7% for biomass content, 50.50% ± 0.20% for protein content and 1.88 g/L ± 4.9% for residual sugar content.

The acquired results revealed that the mathematical models obtained are able to generate the best values for biomass gain and protein and residual sugar contents.

ABSTRACT

Fodder yeasts are successfully utilized to feed animals since they are considered a rich source of well digested protein and vitamins.

The study aimed to find new carbon sources for fodder yeast development with viable economic effects while reducing the pollution to the environment by capitalisation of wastewaters with high monosaccharides content from pulp and paper industry and by employing a hydrolytic product obtained from sugar beet pulp. *Candida utilis* yeast strain was used as inoculum.

The fabrication recipe was established with the help of Response Surface Methodology by optimising the amount of ingredients and having as response functions protein, biomass and residual sugar. The found optimized values were: 24 g/L zinc reducing sugar, 1038 mg/L nitrogen and 420 mg/L phosphorous. In these conditions the final product had 50.98% protein content, w/w and 6.49 g/L biomass, w/w with a consumption of reducing sugar of 92.66%.

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