OPTIMIZATION OF PROTEIN PRODUCTION BY *GEOTRICHUM CANDIDUM* MIUG 2.15 BY CULTIVATION ON PAPER RESIDUES, USING RESPONSE SURFACE METHODOLOGY

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Response surface methodology (RSM) based on the 2^3 factorial central composite design (CCD) was used to optimize the biotechnological conditions for growth and protein production by a selected fungal strain *Geotrichum candidum* MIUG 2.15, by solid-state cultivation on a semisolid medium based on a mixture of paper residues, *i.e.* office paper, newspaper, and cardboard, mixed in a ratio of 1:1:1(w/w), supplemented with cheese whey waste and complex manure. Three independent variables, the solid:liquid ratio, the concentration of complex manure, and cultivation time, were evaluated to determine their correlative effect on biomass production and protein biosynthesis. The optimal conditions for obtaining a maximum protein yield of 9.53% w/w dry mass were the following: the complex manure concentration of 0.5%, the solid:liquid ratio of 1:5, and the growth time of 10 days.

Keywords: Geotrichum candidum; Protein production; Solid-state fermentation; Paper residues; Response surface methodology (RSM)

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INTRODUCTION

Bioconversion processes have been developed for the utilization of renewable resources to produce useful chemicals and feed stocks. The use of renewable resources and, in particular, of hemicellulosic biomass, as substrate in biotechnological processes has broad economic and environmental implications.

Lignocellulosic wastes from different sources have varying composition of hemicellulose, cellulose, and lignin. Some sources of lignocellulosic material are wood from angiosperms and gymnosperms, grasses, leaves, wastes from paper manufacture, sugarcane bagasse, wheat straw, wheat bran, rice bran, groundnut shell, and other agricultural wastes (Bahrim 2004).

Fungal single cell protein (SCP) production can be a very attractive alternative to valorize a variety of agro-industrial by-products and municipal wastes, including molasses, hydrocarbons, lignocellulosic materials, waste from wood industry, paper residues, cheese whey waste, wastewaters, *etc*.

Few applications for SCP production have been realized at the laboratory and pilot-scale levels by using paper residues as fermentative substrate. The composition of paper is from 40% to 80% cellulose, 20% to 30% lignin, and 10% to 30% hemicellulose and xylosans (www.fao.org).

Prior to use, the paper residues need preliminary treatment; the polymers are hydrolyzed in order to obtain simple components which can be then easily metabolized by cultivated microorganisms (Leuștean *et al.* 2012). After hydrolysis, carbon is the main chemical component. For this reason, mixing the paper residues with organic and inorganic nitrogen sources (cheese whey waste, complex manure, *etc.*) contributes to balancing the composition of the fermentative medium from a nutritional point of view. Whey contains carbon and nitrogen compounds assumable by the microorganisms and is widely available. Therefore it could be used for the production of microbial biomass. Valorization of whey is also interesting because its chemical oxygen demand is elevated by high concentrations of soluble material that are extremely polluting for the environment, and there is an associated need to protect the environment from the extremely large quantities of whey that are generated by the industrial production of cheese (Spălățelu 2012).

The protein obtained from the microorganisms is not only cheap but also may provide a balanced nutrition for humans and animals (Rajoka *et al*. 2006). SCP is normally considered to be a valuable source of protein, but it also contains nucleic acids, carbohydrates, cell wall material, lipids, minerals, and vitamins (Ugalde and Castrillo 2005).

The valorization of paper residues and cheese whey waste as abundant and low-cost raw materials in the production of fungal biomass which could be used as a protein supplement (SCP) for animal feed production would not only be economically viable but would also solve problems caused by the accumulation of organic wastes and protect the environment (Pandey 2003; Silva *et al.* 2011).

Most of the research on SCP production has been focused on the use of the yeast *Candida utilis* (formerly *Torulopsis utilis*, *Torula utilis*) strains. The mold *Geotrichum candidum* has also been the subject of biochemical and physiological studies due to common occurrence and its biotechnological value. The strains are able to grow on different substrates and produce many enzymes such as: cellulases, xylanases, lipase, proteases, or peroxidases (lignin peroxidase and manganese peroxidase) (Boutrou and Guéguen 2005; Witkowska and Piegza 2006; Asses *et al*. 2009). The production of enzymes varies greatly from one strain to another. Based on this multiple enzymatic potential, *Geotrichum candidum* strains are able to grow well on solid and liquid organic wastes in order to obtain organic decontamination and also to produce fungal biomass rich in proteins (Zara 1999; Asses *et al*. 2009).

Response surface methodology is a three-factorial design method, which provides the relationship between one or more measured dependent responses and a number of input (independent) factors. The response surface method is advantageous because it requires a small number of experiments, it is suitable for multiple factor experiments, it seeks relativity between multiple factor experiments, and it finds the most suitable correlation and forecast. Therefore, it finds the optimum values of the factors under investigation, and it predicts the response to the optimum conditions (Popa *et al*. 2007).

Limitations of the single-factor optimization can be eliminated by employing response surface methodology (RSM), which is used to explain the combined effects of all the factors in a fermentation process (Popa *et al*. 2007; Zheng *et al*. 2008; Montgomery 2005).

In the present work, a central composite design (CCD) of response surface methodology (RSM) has been used to optimize the biotechnological parameters of the

solid-state growth and protein biosynthesis for *Geotrichum candidum* MIUG 2.15 strain by cultivation in stationary conditions on a semisolid medium based on paper residues supplemented with cheese whey waste and complex manure.

EXPERIMENTAL

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich and used without further purification. The complex manure containing N-P-K, in ratio of 7:4:5 w/v, used to enhance the nutritive value of the substrate, is a commercial biofertilizer purchased from a specialized market from Galati, Romania. Cheese whey waste was obtained from Galacta SA dairy factory from Galati, Romania.

Mold strain

Geotrichum candidum MIUG 2.15 strain is member of the Collection of microorganisms of Bioaliment Research Platform of "Dunărea de Jos" University of Galati, Romania. It was maintained on agar slants supplemented with (% w/v): glucose 2, peptone 1, yeast extract 0.5, and agar 2, pH = 5.0, and kept at 4 °C.

Paper residues treatment

The cellulosic waste materials used as the solid substrate of the culture medium consisted of a mixture of three components based on paper residues: office paper, newspaper, and cardboard, mixed in a ratio of 1:1:1 (w/w). The materials used were milled using a vibratory ball mill (Janke and Kukel GmbH and Co. Ika Labortechnik), and dried to 80% dry matter, at 60 °C, for 6 hours, using a laboratory drying oven (MMM Group, GmbH Germany). The chemical characteristics of waste cellulosic material used as substrate were determined according to standard procedures in accordance with the AOAC method (Table 1).

Table 1. The Chemical Composition of Cellulosic Waste Substrate Based on Paper Residues

Type of paper residues	Cellulose % w/w	Hemicellulose % w/w	Lignin % w/w	Ash % w/w
Office paper	85	0	15	7.11
Newspaper	61	16	21	7.23
Cardboard	60	20	20	8.56

Culture conditions and protein yield determination

A semisolid medium was prepared, containing 4 g of milled untreated mixed cellulosic material based on three paper residues and cheese whey waste in a solid:liquid ratio varying between 1:3.3 and 1:6.7. It was then supplemented with complex manure, containing 33.5% total nitrogen (16.7% ammonium nitrogen and 16.8% nitrogen in

nitrates). The physico-chemical characteristics of whey were evaluated using Milk-Lab UK Ltd (Oldham, UK) and are shown in Table 2.

Table 2. Physico-chemical Characteristics of Whey

Parameter	Unit	Value
Fat	%	0
Non-fat dry substance	%	7.16
Protein	%	2.84
Lactose	%	3.72
Ash	%	0.59
Density	g/cm^3	1.026
pH		6.0
Cryogenic point	°C	-0.412

The medium was prepared in Petri dishes and was then sterilized at 120 °C for 30 minutes. The sterile medium was inoculated with 10^7 CFU/g substrate suspension of *Geotrichum candidum* MIUG 2.15 conidiospores, and then the cultivation was performed in stationary conditions at 20 °C. After cultivation, the total nitrogen content (N) expressed as % w/w dry mass of solid-state fermented crude product was measured by using the Kjeldahl system (VELP, Italia). The crude protein value was expressed as N x 6.25. The assays were performed in duplicate.

Response surface methodology for the optimization of protein production

A factorial central composite design with three factors, at the central point and star points, was used for the investigation (Agaie *et al*. 2009). The used independent variables were: complex manure concentration as inorganic nitrogen and phosphorous source, solid:liquid ratio, and time of cultivation, each at five coded levels (-α, -1, 0, +1, +α) as shown in Table 3. The response value (Y) in each trial is the average of duplicates.

Table 3. The Independent Variables and Their Levels for the Central Composite Experimental Design

Variables	Code	Coded level of variables				
		-α	-1	0	+1	+ α
Complex manure concentration, %	A	0	0.1	0.3	0.5	0.63
Solid:liquid ratio	B	1:3.3	1:4	1:5	1:6	1:6.7
Time of cultivation, days	C	4	6	8	10	12

The chosen independent variables used in this experiment were coded according to the following equation,

$$x_i = (X_i - X_o) / \Delta X, \text{ for } i = 1, 2, \ldots, k \quad (1)$$

where, x_i is the dimensionless value of a variable, X_i the actual value of a variable, X_0 the value of x_i at the center point, and ΔX is the step change.

The second order polynomial coefficients were calculated and analyzed using the 'Design Expert' software statistical package (www.statease.com). The general form of the second degree polynomial Eq. (2) is:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=1}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

where, Y is the predicted response, X_i and X_j are the input variables, β_0 is the intercept term, β_i is the linear effect, β_{ii} is the squared effect, and β_{ij} is the interaction term.

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the Fisher's F-test (overall model significance), its associated probability $p(F)$, the correlation coefficient R, and the determination coefficient R^2 which measures how well the regression model fits the data. For each variable, the quadratic models were represented as contour plots (3D), and response surface curves were generated using Design Expert 7.0 software version 6.0, Stat-Ease Inc., Minneapolis, USA (Kim *et al*. 2008).

RESULTS AND DISCUSSION

Previous studies based on the experimental Plackett–Burman design made it possible to verify the variables that had an effect on paper residues biotransformation. Among the variables tested, the solid:liquid ratio in the fermentative medium composition has a positive influence, because the optimum water content is essential for the growth and metabolic activity of the microorganism (Leuștean *et al.* 2010).

The physiological and biochemical characteristics of *Geotrichum candidum* MIUG 2.15 strain on cultivation on media based on whey were also investigated previously (Zara 1999; Palela *et al.* 2008, 2010).

In the present study, the correlative effect of three variables, solid:liquid ratio of the semisolid medium, concentration of complex manure, and time of cultivation, upon *Geotrichum candidum* MIUG 2.15 growth and protein production by cultivation in solid-state system, were investigated by statistical optimization using response surface methodology (RSM), in order to find the optimum conditions to increase the crude protein content of the final fermented product.

The Central Composite Design consisted of 15 experimental trials and is shown in Table 4.

The final model equation is shown as (Eq. 3):

$$Y = 4.02 - 0{,}055A + 0.80B - 0.98C - 1.49AB + 1.42AC - 0.23BC +$$

$$0.11A^2 + 0.31B^2 + 0.55C^2 + 1.35ABC - 0.17A^2B + 1.23A^2C \quad (3)$$

The statistical significance of the model equation was checked using F-test analysis of variance (ANOVA). The fitness of the models was also expressed by the coefficient of determination, R^2, for the quadratic model, which was found to be 0.9797 for the production of protein. This value indicates there was 97.97% of response variability in protein production.

Table 4. The Experimental Design of the Biotechnological Conditions (Independent Variables) of Protein Production (Response) by Cultivation of *Geotrichum candidum* MIUG 2.15 Strain in Solid State Fermentation System on Cellulosic Waste Based on Paper Residues

Run	Complex manure concentration, %		Solid:Liquid ratio		Time of cultivation, days		Protein concentration, experimental values, % w/w dry mass	Protein concentration, predicted values, % w/w dry mass
	Coded level	Value	Coded level	Value	Coded level	Value		
1	-1	0.1	1	1:6	-1	6	12.079	12.079
2	-1	0.1	-1	1:4	-1	6	4.660	4.660
3	1	0.5	-1	1:4	1	10	9.066	9.066
4	0	0.3	0	1:5	0	8	2.978	4.020
5	0	0.3	-1.68	1:3.3	0	8	3.546	3.550
6	-1	0.1	-1	1:4	1	10	5.500	5.500
7	-1.68	0	0	1:5	0	8	4.415	4.420
8	1	0.5	-1	1:4	-1	6	7.977	7.980
9	0	0.3	1.68	1:7	0	8	6.250	6.250
10	1	0.5	1	1:6.7	1	10	9.608	9.610
11	0	0.3	0	1:5	1.68	12	3.942	3.940
12	1.68	0.7	0	1:5	0	8	4.230	4.230
13	-1	0.1	1	1:6	1	10	6.570	6.570
14	0	0.3	0	1:5	0	8	4.163	4.020
15	1	0.5	1	1:6	-1	6	4.030	4.030
16	0	0.3	0	1:5	-1.68	4	7.228	7.230
17	0	0.3	0	1:5	0	8	3.987	4.020
18	0	0.3	0	1:5	0	8	3.640	4.020
19	0	0.3	0	1:5	0	8	4.374	4.020
20	0	0.3	0	1:5	0	8	4.951	4.020

Table 5. Analysis of Variance (ANOVA)

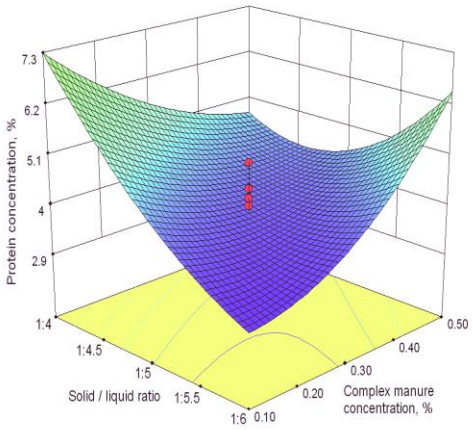
Source	Sum of squares	Degrees of freedom	Mean squares	F-value	P-value
Model	108.320	14	7.740	17.24	0.0027
A	0.017	1	0.017	0.04	0.8529
B	5.400	1	5.400	12.03	0.0179
C	7.180	1	1.000	7.18	248.26
A^2	0.140	1	0.140	0.32	0.5988
B^2	1.170	1	1.170	2.60	0.1676
C^2	3.690	1	3.690	8.23	0.0350
AB	17.680	1	17.680	39.41	0.0015
AC	16.060	1	16.060	35.80	0.0019
BC	0.430	1	0.430	0.96	0.3713
ABC	14.680	1	14.680	32.72	0.0023
A^2B	0.094	1	0.094	0.21	0.6664
A^2C	4.990	1	4.990	11.11	0.0207
AB^2	0.280	1	0.280	0.62	0.4678
A^2B^2	19.100	1	19.100	42.56	0.0013
Pure error	2.240	5	0.450	-	-
Total	110.560	19	-	-	

The Model F-value of 17.24 implies the model is significant. There is only a 0.27% chance that a "Model F-Value" this large could occur due to noise. "Model P-value" less than 0.0500 indicates that the model terms are significant. In this case B, C, AB, AC, C^2, ABC, A^2C, and A^2B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms, the model reduction may improve the model. The significant lack of fit is considered to be bad if one wants the model to fit (Table 5).

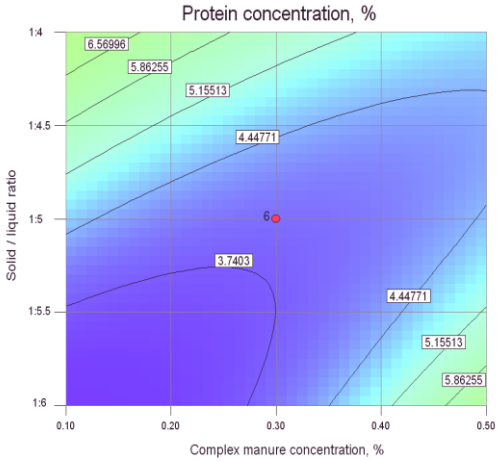
In Table 6, the negative "Predicted R-Squared" implies that the overall average is a better predictor of the response than the current model. The "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 14.709 indicates an adequate signal.

Table 6. Statistic Parameters for Central Composite Design Model

Standard Deviation	0.67	R-Squared	0.9797
Mean	5.66	Adjusted R-Squared	0.9229
C.V. %	11.84	Predicted R-Squared	negative
PRESS	negative	Adequate Precision	14.709



(a)

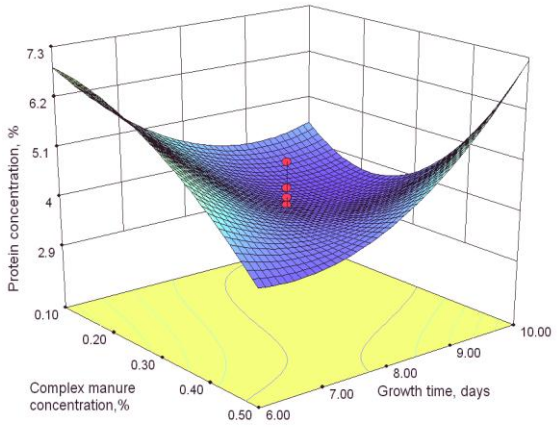


(b)

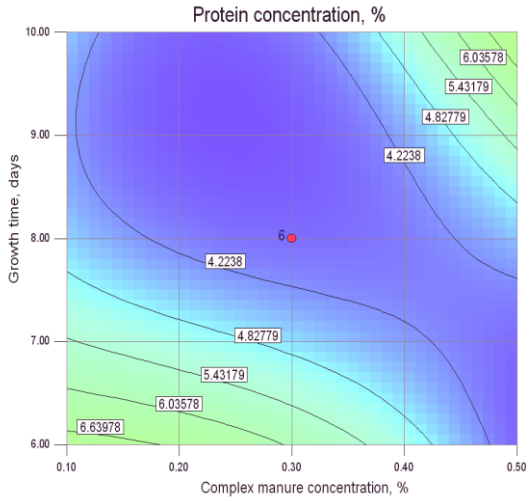
Fig.1. Response surface plot (a) and contour plot (b) showing the effect of complex manure concentration and solid:liquid ratio on protein production by *Geotrichum candidum*

As can be seen in Fig. 1 (a, b), the protein yield increased when the complex manure concentration increased to 0.5%**,** and when the solid:liquid ratio increased up to the level of 1:4.

A small increase can be observed in Fig. 2 (a, b) for the percentage of 0.5% complex manure concentration over a time of cultivation of 10 days.



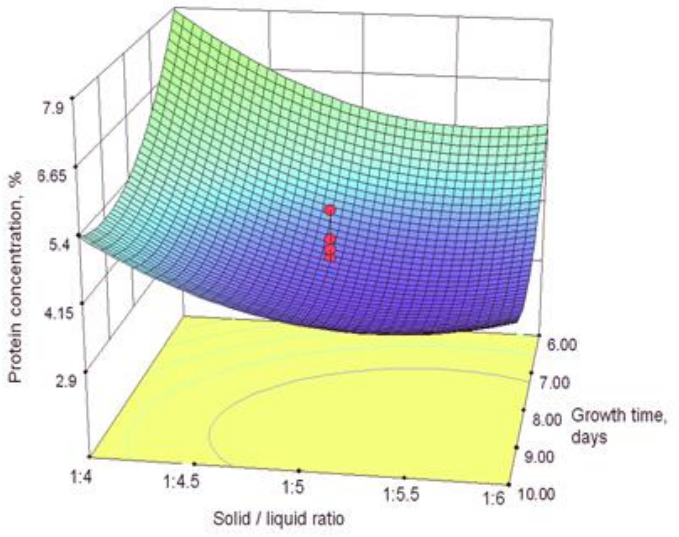
(a)



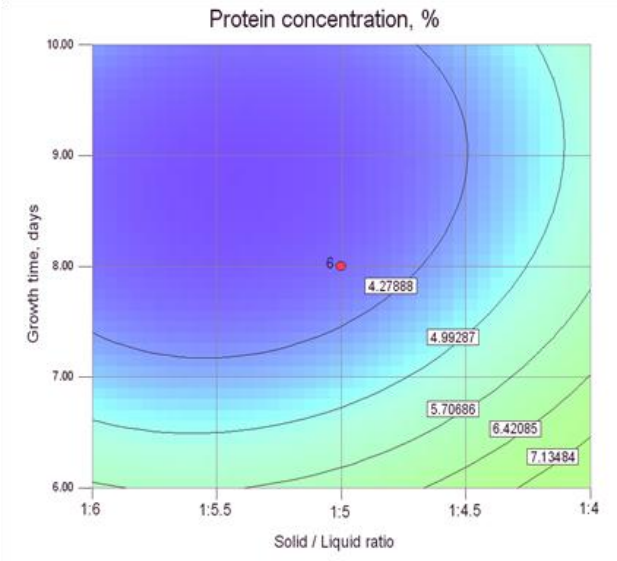
(b)

Fig. 2. Response surface plot (a) and contour plot (b) showing the effect of complex manure concentration and time of cultivation on protein production by *Geotrichum candidum* MIUG 2.15

Figure 3 (a, b) shows that the protein content increased at a solid:liquid ratio of 1:4 and a cultivation time of 6 days. The contour plots shows that two by two, the factors influence each other, the complex manure concentration, the solid:liquid ratio and time of cultivation.



(a)



(b)

Fig. 3. Response surface plot (a) and contour plot (b) showing the effect of the time of cultivation and solid:liquid ratio on protein production by *Geotrichum candidum* MIUG 2.15

Figure 4 also shows that the protein production was influenced by the time of cultivation (C) and by the solid:liquid ratio. The corresponding slopes are more pronounced and much more extended compared to those related to complex manure concentration, which are almost linear.

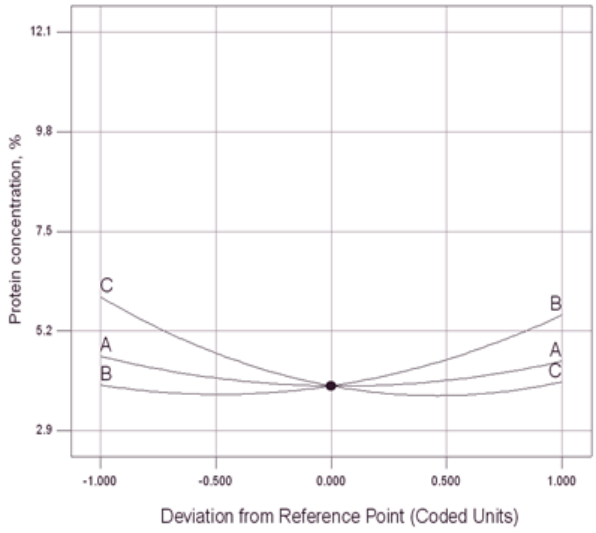


Fig. 4. Perturbation evolution for three factors involved in protein production by *Geotrichum candidum* MIUG 2.15. A – complex manure concentration, B – solid:liquid ratio, C – time of cultivation

Analyzing the results in the cube representation (Fig. 5), one can see a maximal protein yield (12.079 % w/w dry biomass) corresponding to the following conditions of cultivation conditions: 0.1% complex manure concentration, solid:liquid ratio of 1:4**,** and time of cultivation of 6 days.

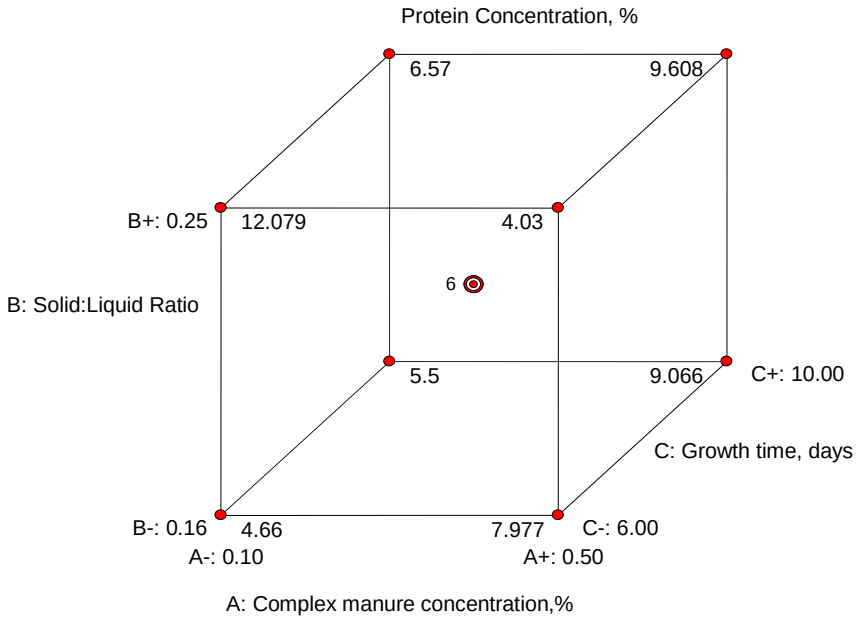


Fig. 5. Cube plot showing the influence of the relevant factors involved in the protein production by *Geotrichum candidum* MIUG 2.15 in solid-state fermentation system on paper residues A – complex manure concentration, B – solid:liquid ratio, C – time of cultivation

The small differences between the experimental data and the prediction of the model chosen can be observed (Fig. 6).

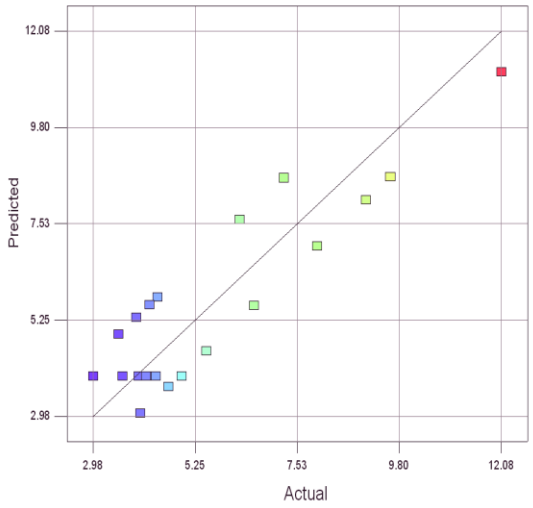


Fig. 6. Parity plot showing the distribution of experimental vs. predicted values

The numerical method (www.statease.com) was used to solve the regression equation. The model validation was conducted using the same bioconversion conditions, as well as the comparative model to the predicted values (Table 7).

Table 7. Model Validation (Biotechnological Conditions, Responses) For Protein Production by *Geotrichum candidum* MIUG 2.15 by Cultivation in Solid State Fermentation System on Paper Residues

Run	Complex manure concentration, %	Solid:liquid ratio	Time of cultivation, days	Experimental yield of protein values, % w/w dry mass	Predicted yield of protein values, % w/w dry mass
1	0.1	1:3	6	2.92	2.82
2	0.3	1:4	8	4.98	5.13
3	0.5	1:5	10	9.53	9.066

To confirm the predicted optimum biotechnological conditions for protein production by *Geotrichum candidum* MIUG 2.15 by solid-state cultivation on paper residues, the theoretical optimum conditions were used. The model predicted that the maximum yield of protein would be 9.066 % w/w dry mass. The experimental results of 9.53 % w/w dry mass were in close agreement with the model prediction. The predicted yield of protein value was calculated through the model equation (Eq. 3).

Rao *et al*. (2010) reported obtaining of 46% microbial protein production by cultivation of *Penicillium janthinellum* selected strain on medium containing bagasse hydrolysate, ammonium sulphate, and potassium dihydrogen phosphate. Miller and Srinivasan (1983) obtained 23 to 38% protein content of biomass by cultivation of a thermotolerant strain of *Aspergillus terreus* on cellulose with a rate of biotransformation of the substrate of 78 to 84%. Khan and Dahot (2010) investigated the effect of

agricultural wastes (sugar cane bagasse, orange peel, wheat straw, and rice husk), some plant seeds (cotton seeds, cajanus cajan seeds, and castor beans), and pure sugars (mannose, glucose, fructose, galactose, maltose, lactose, lactose, sucrose, starch, cellulose) on the production of SCP by *Penicillium expansum* strain. The maximum yields of biomass (1.64 g/l) and protein (18.25%) were obtained when rice husk was used as carbon source. Higher yields of biomass (1.92 g/l) and protein (21.36%) production were obtained when acid hydrolysates of rice husk and cotton seeds were mixed.

Although the results reported in the literature relating fungal crude protein production by biovalorisation of agro-industrial wastes are higher than those highlighted in this study, our study is original compared with the few data existing in scientific literature, regarding the wastes and fungus strains used in biotechnological processes. Therefore, the biotechnological process proposed by this study is very important because the paper residues are used as solid substrate without other treatments (pretreatments, chemical or enzymatic saccharification), the *Geotrichum candidum* MIUG 2.15 strain has a good enzymatic potential and whey and complex manure play an important role in fungal metabolic activity stimulation.

The utilization of two abundant, recalcitrant, and low-cost raw materials (paper residues and whey) for the production of crude protein has great economic impact for high grade animal feed production and it also solves problems caused by organic wastes accumulation and environmental pollution.

CONCLUSIONS

The growth ability and protein biosynthesis of *Geotrichum candidum* MIUG 2.15 strain by solid-state cultivation on a medium based on a mix of three paper residues and whey were studied. The following conclusions were drawn from the results:

1. The statistical optimization of growth of the mold strain on paper residues in the solid-state fermentation system has been successfully carried out by using RSM**,** based on the 2^3 factorial CCD, in order to increase the yield of proteins in controlled biotechnological conditions, such as time of cultivation, complex manure concentration, and solid:liquid ratio.

2. The optimal conditions for obtaining a maximum protein yield of 9.53% w/w dry mass were the following: a complex manure concentration of 0.5%, a solid:liquid ratio of 1:5, and a cultivation time of 10 days.

3. After cultivating the *Geotrichum candidum* MIUG 2.15 strain on the paper residue substrate supplemented with whey and complex manure, the cellulose content was 53% lower, while the protein content was increased by 6.7%.

4. The results suggest that the paper residues supplemented with cheese whey waste could be valuable substrates for SCP production with the *Geotrichum candidum* selected strains, resulting in a fermented product with useful application in animal feeding and that could also be a good opportunity to minimize the environmental pollution caused by the agro-industrial and municipal by-products.

ACKNOWLEDGMENTS

The authors acknowledge material and financial assistance from the *Integrated Center for Research and Education, Biotechnology Applied in Food Industry – Bioaliment* (www.bioaliment.ugal.ro)

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Article submitted: March 30, 2012; Peer review completed: August 9, 2012; Revised version received: August 30, 2012; Accepted: August 31, 2012; Published: September 12, 2012.