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Optimizing Biotechnological Production of Glucosamine as Food Ingredient from *Aspergillus* sp. BCRC 31742

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Abstract: The screening of medium constituents for Glucosamine (GlcN) production was investigated. Culture conditions, C source, N source, C/N ratio and macro-mineral concentration were investigated in preliminary studies. The white fine sugar was the best carbon source resulted which is inexpensive as compared with glucose. The medium constituents were optimized using response surface methodology. White fine sugar and peptone concentration were finally optimized using central composite design. GlcN concentration produced experimentally using optimized medium constituents was 5.48 g L^{-1} which is the highest in the literature using wild fungi cultivated in flask. Moreover, cultivation in fermenter resulted in GlcN concentration of 3.91 g L^{-1} with biomass of 14.6 g L^{-1} and productivity of 23.3 mg L^{-1} h.

Key words: Glucosamine, Aspergillus, response surface methodology, fermenter, fungi, cultivation

INTRODUCTION

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) is a naturally occurring amino monosaccharide that is endogenously formed throughout human body by cellular glucose metabolism (D'Ambrosio *et al*., 1981). GlcN mainly GlcN hydrochloride (GlcN-HCl) which is discussed herein have been being utilized as dietary supplement and subjected to alleviation of pain and inflammation in joints from osteoarthritis as well as reducing joint space narrowing. However, recently in addition to dietary supplement, according to GRAS Notice No. GRN 000150, further explained in 21 CFR 170.3(n) (3), (7), (16), (31), (36), GlcN-HCl can be used as food ingredient in various food products as described in 21 CFR 8 170.3-Broad food categories and USDA's CSFII-Food categories (Mattia, 2004; Rogers, 2004).

Natural resources of GlcN can be extracted through chemical and enzymatic hydrolysis of crustaceans (i.e., shrimp, crab and lobster), plants, yeast and fungi or even produced as secondary metabolite using *E. coli* (Deng *et al*., 2005; Ferrer *et al*., 1996; Jung *et al*., 2005; Kuk *et al*., 2005; Rattanakit *et al*., 2003; Sashiwa *et al*., 2002). Especially in fungi, fungal biomass resulted from various fermentations can be used as the source of chitin or chitosan to be hydrolyzed into GlcN (Bowman and Free, 2006; Cao *et al*., 2008; Kim *et al*., 2001; Nwe *et al*., 2002). Deposition of chitin content in fungal cell wall found to be different for each strain of fungus being utilized and also depends on both culture conditions and cultivation medium (Chatterjee *et al*., 2005).

Several literatures have been reported on GlcN production using fungi under different cultivation conditions (Carter *et al*., 2004; Hsieh *et al*., 2007; Liao *et al*., 2008; Ruiz-Teran and Owens, 1996; Sparringa and Owens, 1999; Yu *et al*., 2005). GlcN production is frequently coupled with other intended products such as enzymes, organic acids and other secondary metabolites as fine chemicals. Since GlcN production depends on fungal biomass concentration, its production will confidently depend on culture conditions and medium constituents. There was no scientific paper been reported on optimizing the medium constituents of fungal fermentation to produce GlcN. Hence, this study aims to investigate optimum conditions for enhancing GlcN production biotechnologically.

There are many statistical approaches can be used for optimizing medium constituents, such as One Variable At a Time (OVAT), orthogonal matrix method, partial factorial, central composite design, multiple linear regression, response surface methodology (Gbolagade *et al*., 2006; Hajji *et al*., 2008; Kennedy and Krouse, 1999; Lim *et al*., 2004). Among them, Response Surface Methodology (RSM) is a suitable one for

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identifying the effect of individual variable and for seeking the optimum conditions for a multivariable system efficiently (Box and Draper, 1987; Ratnam *et al*., 2005). In this study optimization of medium constituents were carried out using response surface methodology whereas for culture conditions OVAT would be utilized. Two-level full factorial design was employed for screening important factors resulted from preliminary studies (in case of medium constituents such as white fine granulated sugar, peptone, $ZnSO_4$, $MnSO_4$). Moreover, only white fine granulated sugar and peptone concentration were optimized using RSM by means of steepest ascent and canonical analysis to give a response (yield) as a surface. Furthermore optimized conditions resulted from RSM data then applied in fermenter of 5 L working volume. DO value was taken into account. Several aspects were also studied in this cultivation.

MATERIALS AND METHODS

Microorganism: *Aspergillus* sp. BCRC 31742 was purchased from Bio-resource Collection and Research Center (BCRC), Taiwan. The strain was cultured on potato dextrose agar (PDA-Difco, 39 g L^{-1}) and incubated at 30°C for 7 days. For pretreatment, spores resulted from PDA were collected and suspended with distilled water then transferred quantitatively to a potato dextrose broth (PDB, 24 g L^{-1}), incubated at 30°C and shaken at 200 rpm for 7 days. Preservation was carried out by putting 0.5 mL spores suspension (mycelia, clumps and pellets) from broth and 0.5 mL glycerol (50% v/v) into eppendorf and finally stored at -80°C.

Materials: The GlcN standard (D-(+)-GlcN hydrochloride, 99% in purity), 1-napthyl isothiocyanate (98% in purity) and 3,5-dinitrobenzoacetonitrile (97% in purity) were purchased from Sigma, USA. Reagent pyridine (99.5% in purity) and acetonitrile of HPLC grade (99.8% in purity) were bought from Riedel-de Haen and Mallinckrodt Chemicals, respectively. A medium contained superior White Fine (WF) granulated sugar (Taiwan Sugar Corporation, Taiwan; 33.9 g L^{-1}), mycological peptone (DIFCO, USA; 40.6 g L^{-1}), KH_2PO_4 (R.D.H, Germany; 0.5 g L^{-1}), $MgSO_4.7H_2O$ (R.D.H, Germany; 0.5 g L^{-1}) and $CaCl_2.2H_2O$ (Yakuri, Japan; 0.1 g L^{-1}).

Fungal fermentation: Firstly, preserved fungi were activated using PDB (24 g L^{-1}) for 5 days at 30°C, shaken at 200 rpm. Then spores, pellets and clumps of *Aspergillus* sp. BCRC 31742 (10% v/v, 0.23±0.001 gdw seed) were transferred into initial GP medium (glucose, 25 g L^{-1}; peptone, 20 g L^{-1}; KH_2PO_4, 0.5 g L^{-1}; $MgSO_4$, 0.5 g L^{-1}; $CaCl_2$, 0.1 g L^{-1}). For preliminary study we have investigated several fermentation aspects such as agitation, pH, incubation temperature. Therefore, for optimizing medium constituents using RSM, cultivation would be carried out in 250 mL flask, shaken at 200 rpm, pH 7.0 and incubated for 7 days. These values had been well explained and even were agreeable according to several published literatures (Braun and Vecht-Lifshitz, 1991; Li *et al*., 2002; Papagianni, 2004; Xiong *et al*., 2005).

Determination of fungal GlcN: Determination of fungal GlcN was carried out by means of HPLC and adopted from the previous studies (Hsieh *et al*., 2007; Sitanggang *et al*., 2009) wherein hydrochlorination process used conventional thermal method. The analytical HPLC column was a Li Chrospher® 100 RP-18 endcapped (5 μm, 4 mm i.d.×250 mm) column. This reversed phase column is shipped in acetonitrile-water. Detector used was UV-Vis detector SPD-10 A, 0.0100 AUFS (Shimadzu, Japan). Pressure was maintained at 130-150 kgf. The mobile phase solvent a was HPLC-grade water and solvent B was acetonitrile (HPLC grade, 99.8% in purity). The ratio between water to acetonitrile was 87:13. The temperature of column was maintained at 40°C with a mobile phase flow rate of 1.3 mL min^{-1}. Detection was performed at a wavelength (λ) of 230 nm with an analytical time of 40 min.

Medium optimization using response surface methodology: In many engineering fields, there is a relationship between an output variable of interest y and a set of controllable variables $\{x_1, x_2, \ldots, x_n\}$. The nature of the relationship between y and x values might be known for some systems. Then a model can be written in the form:

$$y = f\{x_1, x_2, \ldots, x_n\}+\varepsilon$$

where, ε represents noise or error observed in the response y. If we denote the expected response by $E(y) = f\{x_1, x_2, \ldots, x_n\} = \omega$, then the surface can be represented by:

$$\hat{y} = f\{x_1, x_2, \ldots, x_n\}+\Sigma \quad (1)$$

This formula is called response surface. In most of the RSM problems, the form of relationship between response and independent variable is unknown. Thus the first step in RSM is to find a suitable approximation for the

true functional relationship between y and set of independent variables employed. The results of central composite design are usually used to fit a second order polynomial equation as it presents in the behavior of such systems. A second order model is usually utilized in response surface methodology as follows:

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

Where:

$\hat{y}$	=	Predicted response
β_0	=	Offset term
β_1 , β_2 , β_3, β_4	=	Linear effects
β_{11} , β_{22} , β_{33} , β_{44}	=	Squared effects
β_{12} , β_{13} , β_{14} , β_{23} , β_{24} , β_{34}	=	Interaction effects
x_1	=	First factor
x_2	=	Second factor and so on

Using more factors from a process would just require larger order interactions in the equation. This phenomenon is called a quadratic model (Montgomery, 1991).

RESULTS AND DISCUSSION

Effects of C source, N source, C/N ratio and macro-mineral on GlcN production: Two kinds of carbon source were utilized such as carbohydrate and oil based compounds. For C source from carbohydrate compounds, there were ten compounds (brown sugar, WC sugar, WF sugar, fructose, glucose, maltose, mannitol, molasses, soluble starch, sucrose) being studied whereas for oil there were six compounds (sunflower oil, peanut oil, olive oil, soybean oil, glycerol and sesame oil) were investigaed. Figure 1a and b show that utilization of carbohydrate based compounds for C source resulted higher GlcN fermentation performances as compared to oil ones. Particularly in carbohydrate based compounds, GlcN concentrations for WF sugar and glucose as C source were found almost at the same level, 3.16 and 3.05 g L^{-1}, respectively. Hence, this study concluded to utilize WF sugar as C source instead of glucose regarding to its cost and continued to investigate the effect of its concentration for GlcN fermentation (Fig. 1c). It was found that when WF sugar was 25 g L^{-1}, the GlcN concentration was 3.17 g L^{-1}; biomass concentration, 14.2 g L^{-1}; GlcN content, 0.22 g g^{-1} biomass and productivity, 18.9 mg L^{-1} h.

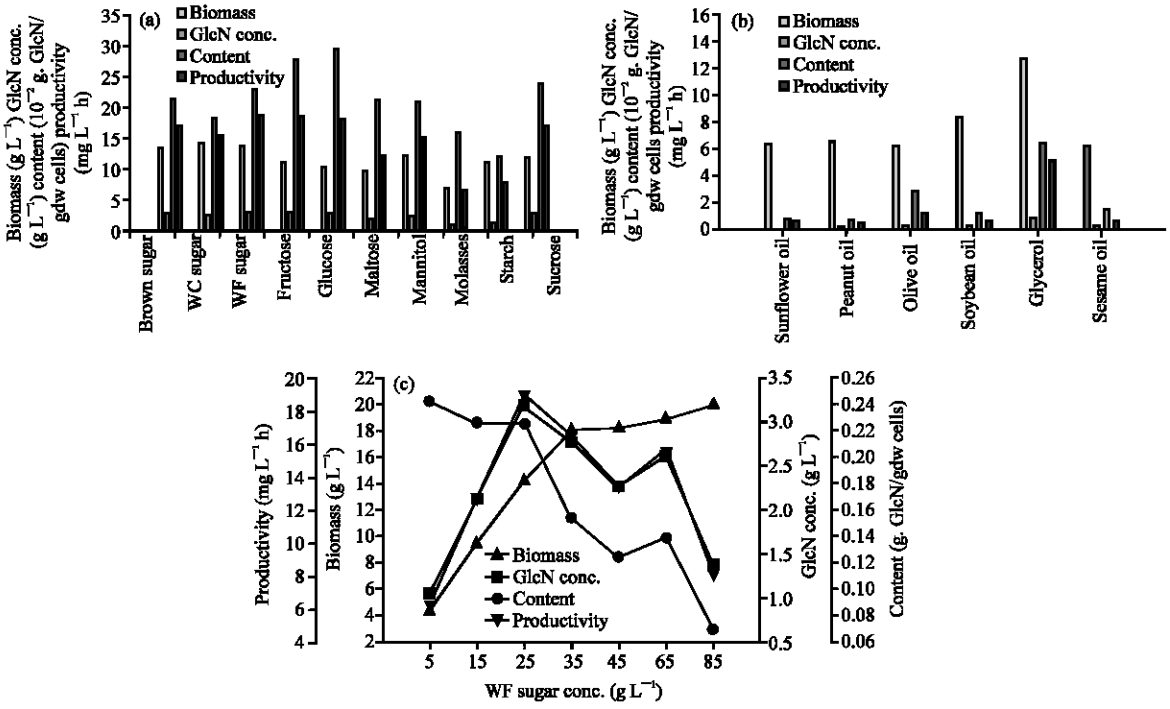

Fig. 1: Effects of various C sources and WF sugar concentration in the medium fermentation of *Aspergillus* sp. BCRC 31742 for producing GlcN. (a) Effect of carbohydrate compounds as C source; (b) Effect of oil compounds as C source; (c) Effect of WF sugar concentration

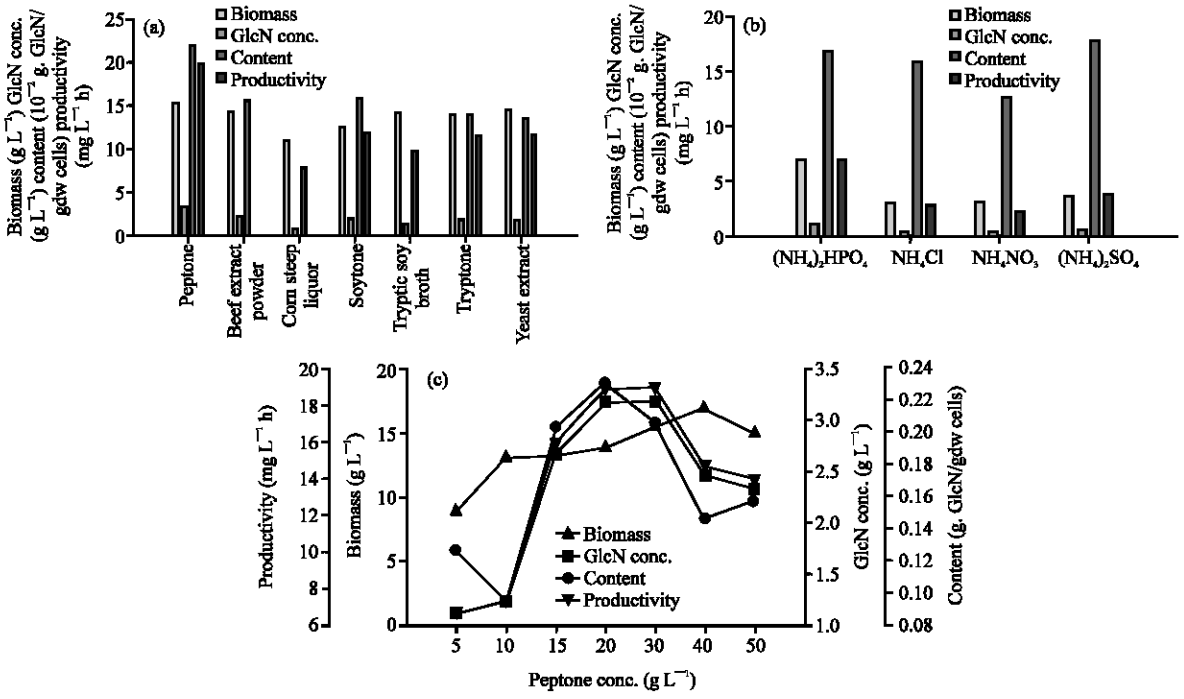

Fig. 2: Effects of various N sources and peptone concentration in the medium fermentation of *Aspergillus* sp. BCRC 31742 for producing GlcN. (a) Effect of organic N source, (b) Effect of inorganic N source, (c) Effect of peptone concentration

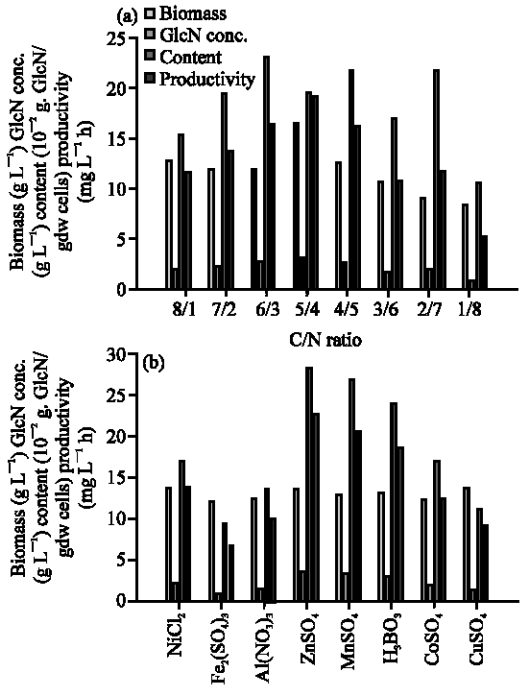

Fig. 3: Effect of C/N ratio and macro-minerals in fermentation medium of *Aspergillus* sp. BCRC 31742 for producing GlcN. (a) Effect of C/N ratio, (b) Effect of various macro-minerals

For N source, peptone was found as suitable material among the others (organic and inorganic N sources) (Fig. 2a-b). When concentration of peptone was 20 g L^{-1}, the GlcN concentration had a maximum value and was 3.16 g L^{-1}; biomass concentration, 13.8 g L^{-1}; content, 0.23 g g^{-1} dw cells and productivity, 18.8 mg L^{-1}. h (Fig. 2c). The study of C/N ratio resulted in optimum C/N ratio of 5/4 for WF sugar and peptone. The results were shown in Fig. 3a. For macro-minerals, 0.1 g L^{-1} of $ZnSO_4$ and $MnSO_4$ were found to be appropriate macro-nutrients for growth of fungi and resulted in higher GlcN concentration (3.79 and 3.44 g L^{-1}, respectively for $ZnSO_4$ and $MnSO_4$) as compared to others (Fig. 3b). Hence, WF sugar and peptone (as C and N source) and macro-nutrients $ZnSO_4$ and $MnSO_4$ were found significantly to affect GlcN production and would be taken as factors to be screened using two-level full factorial design and resulted factors would be optimized using RSM.

Optimization of medium constituents using Response Surface Methodology (RSM): There were four factors (e.g., WF sugar, peptone, $ZnSO_4$ and $MnSO_4$) were initially found to significantly influence GlcN production and considered for further screening using two-level full factorial design before optimization using central

Table 1: Independent variables for experimental plan, coded values and matrix compositions of two-level design of full factorial

Independent variables	Coded levels: -1 (low)	+1(high)
X_1 WF sugar (g L^{-1})	20.0	30.0
X_2 Peptone (g L^{-1})	10.0	30.0
X_3 $ZnSO_4$ (g L^{-1})	0.1	0.5
X_4 $MnSO_4$ (g L^{-1})	0.1	0.5

Pattern	WF sugar	Peptone	$ZnSO_4$	$MnSO_4$	GlcN conc. (g L^{-1})
----	-1	-1	-1	-1	1.95
---+	-1	-1	-1	+1	1.96
--+-	-1	-1	+1	-1	1.89
--++	-1	-1	+1	+1	2.12
-+--	-1	+1	-1	-1	3.32
-+-+	-1	+1	-1	+1	3.95
-++-	-1	+1	+1	-1	2.99
-+++	-1	+1	+1	+1	2.85
+---	+1	-1	-1	-1	2.29
+--+	+1	-1	-1	+1	3.32
+-+-	+1	-1	+1	-1	3.24
+-++	+1	-1	+1	+1	2.46
++--	+1	+1	-1	-1	4.14
++-+	+1	+1	-1	+1	3.93
+++-	+1	+1	+1	-1	3.19
++++	+1	+1	+1	+1	3.51

Table 2: Parameter estimates for regression analysis of GlcN concentration obtained from two-level full factorial design

Term	Estimate*	SE	t-ratio	Prob.>|t|
Intercept	2.882	0.108786	26.49	<0.0001
X_1	0.378	0.108786	3.48	0.0176
X_2	0.477	0.108786	4.39	0.0071
X_3	-0.100	0.108786	-0.92	0.3998
X_4	0.007	0.108786	0.07	0.9506
$X_2 \times X_1$	-0.045	0.108786	-0.42	0.6914
$X_3 \times X_1$	-0.060	0.108786	-0.55	0.6049
$X_3 \times X_2$	-0.124	0.108786	-1.14	0.3053
$X_4 \times X_1$	0.038	0.108786	0.36	0.7654
$X_4 \times X_2$	-0.055	0.108786	-0.51	0.6313
$X_4 \times X_3$	-0.051	0.108786	-0.47	0.6551

*Regression coefficient $R^2 = 0.873$

composite design. Those independent variables were coded and tabulated together with the matrix compositions of two-level design of full factorial in Table 1 including experimental values of GlcN concentration.

Using two-level design of full factorial, a total number of 16 runs with different concentrations of WF sugar, peptone, $ZnSO_4$ and $MnSO_4$ were performed. Each row represents an experiment and each column represents an independent variable. The signs (+) and (-) represented two different levels (highest and lowest value in Table 1) of the each independent variable under investigation. Experimental data collected for GlcN concentration in each run was analyzed using software JMP version 3.2.2 and fitted into a simple linear regression model and resulted in Eq. 3 for GlcN production (for complete decimals and variable refers to Table 2):

$$\hat{y} = 2.882 + 0.378X_1 + 0.477X_2 - 0.100X_3 + 0.007X_4 - \ldots \quad (3)$$

Table 3: Compositions of medium in steepest ascent method

Independent variables	Steps: -1	0	1	2	3	4
X_1 WF sugar (g L^{-1})	21.9	25	28.1	31.2	34.3	37.4
X_2 Peptone (g L^{-1})	12.2	20	27.8	35.6	43.4	51.2

The analytical variance for experimental designs was calculated and the significant level of each medium variable was determined by t-test. Since the R^2 was 0.873, the Eq. 3 was significant model and suitable one to describe the variations between those factors (e.g., superior white fine granulated sugar, peptone, $ZnSO_4$ and $MnSO_4$). From Table 2, it could be seen that both of $ZnSO_4$ and $MnSO_4$ had probability values higher than 5% ($p>0.05$). It was concluded that $ZnSO_4$ and $MnSO_4$ did not have strong interactions and influences unto fermentation medium to affect GlcN production regarding to experimental design. Furthermore, Eq. 3 could be simplified into:

$$\hat{y} = 2.882 + 0.378X_1 + 0.477X_2 \quad (4)$$

Thus, the application of steepest ascent can be made accordingly to Eq. 4 using these gradient equations as following:

$$X_1' = \frac{a_{x1}}{\sqrt{a_{x1}^2 + a_{x2}^2}}.X_1 \quad (5)$$

$$X_2' = \frac{a_{x2}}{\sqrt{a_{x1}^2 + a_{x2}^2}}.X_2 \quad (6)$$

X'_1 and X'_2 were new step value based on basal medium level of WF sugar and peptone concentration, whereas a_{x1} and a_{x2} were coefficient value of X_1 and from Eq. 4 (0.378 and 0.477, respectively). After getting X'_1 and X'_2 value, new medium compositions in steepest ascent were shown in Table 3. The experimental values of GlcN concentration are shown in Fig. 4.

Based on foregoing results of two-level full factorial design and path of steepest ascent, new original points were found for WF sugar and peptone concentration. As shown in Table 4, a central composite design with 13 experiments was performed. The respective low and high level for the factors were defined as 31.2 (-1) and 37.4 (+1) for WF sugar (X_1) and 35.6 (-1) and 51.2 (+1) for peptone (X_2). The final empirical model to predict the glucosamine concentration in system was as following:

$$\hat{y} = 5.384 - 0.195X_1 - 0.689X_2 - 0.622X_1^2 - 0.924X_2^2 \quad (7)$$

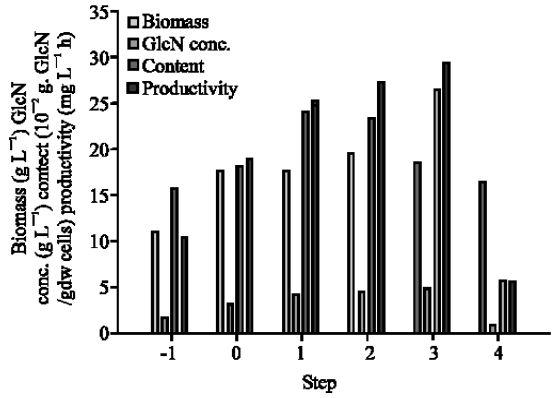

Fig. 4: Plots of GlcN concentration, biomass, GlcN content, productivity of *Aspergillus* sp. BCRC 31742 for producing GlcN as the effect of various WF sugar and peptone concentrations according to steepest ascent method

Table 4: Compositions of medium in central composite design including experimental and predicted GlcN concentration

Run	WF sugar (X_1)	Peptone (X_2)	Exp. GlcN conc.(g L^{-1})	Predicted. GlcN conc.(g L^{-1})
1	-1	-1	4.791	4.722
2	-1	+1	3.665	3.343
3	+1	-1	4.600	4.332
4	+1	+1	3.025	2.953
5	-1.41421	0	4.215	4.416
6	+1.41424	0	3.699	3.864
7	0	-1.41421	4.348	4.510
8	0	+1.41424	2.357	2.560
9	0	0	5.368	5.385
10	0	0	5.476	5.385
11	0	0	5.273	5.385
12	0	0	5.334	5.385
13	0	0	5.472	5.385

Table 5: Parameter estimates of regression analysis of GlcN concentration obtained from central composite design

Term	Estimate*	SE	t-ratio	Prob.>|t|
Intercept	5.384	0.092838	58.00	<0.0001
X_1	-0.195	0.073395	-2.66	0.0325
X_2	-0.689	0.073395	-9.40	<0.0001
$X_1 \times X_1$	-0.622	0.078707	-7.91	<0.0001
$X_2 \times X_1$	-0.112	0.103796	-1.08	0.3154
$X_2 \times X_2$	-0.924	0.078707	-11.75	<0.0001

*Coefficient of determination $R^2 = 0.975$

The suitability of fit of model (Eq. 7) was checked by determination coefficient (R^2). As shown in Table 5, the determination coefficient (R^2) was 0.975 that indicates there only 2.75% of the total variations were not explained by the model (Eq. 7). It proved that the model has a highly significance for explaining the variations. The intercept term, the slope term of X_2 (peptone) and the quadratic square term of $X_1.X_1$ and $X_2.X_2$ were highly significant with a probability of 99% ($p<0.01$); while X_1 (WF sugar) was also significant with probability of 95% ($p<0.05$).

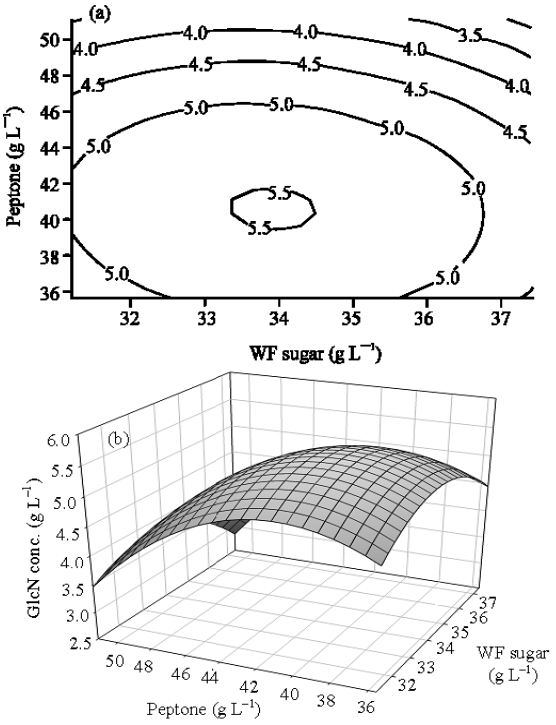

Fig. 5: (a) contour plot and (b) Response surface curve of GlcN concentration as affected by WF sugar and peptone concentration in medium fermentation

However, the interaction term of $X_2.X_1$ had no significant influence on GlcN concentration since the probability was less than 95% ($p>0.05$) (Table 5).

The fitted second-order polynomial equation (Eq. 7) was used to predict the GlcN concentration in the system then depicted as 3D surface and contour plots in order to explore the optimal medium composition (Niladevi *et al*., 2009; Xiong *et al*., 2005). As shown in Fig. 5, the optimal medium compositions (g L^{-1}) with the coded level in parentheses was 33.9 (-0.124) and 40.6 (-0.365) for WF sugar and peptone, respectively. The maximal GlcN concentration under this condition was predicted to be 5.52 g L^{-1}. The confirmatory experiments showed a production of 5.48 g L^{-1} GlcN concentration which was 0.7% lower than the predicted concentration. These medium compositions resulted in a 59.8% increase of GlcN concentration as compared to the previous study and even other studies (Table 6).

Fermenter cultivation: *Aspergillus* sp. BCRC 31742 was cultured using superior white fine granulated sugar, 25 g L^{-1}; Peptone, 20 g L^{-1}; KH_2PO_4, 0.5 g L^{-1}; $MgSO_4$, 0.5 g L^{-1}; $CaCl_2$, 0.1 g L^{-1} in 2 L working volume of 5 L fermenter at 30°C, 200 rpm, pH 7 and cultured for 7 days. When air flow was 1 L m^{-1} without controlling oxygen,

Table 6: Production of GlcN using wild type fungi

Fungus	Biomass (g L^{-1})	GlcN conc. (g L^{-1})	Content (mg g^{-1} dw cells)	Productivity (mg L^{-1} h)	GlcN yield (mg g^{-1}-carbon)	References
Rhizopus oligosporus NRRL 2710	-	-	0.11	-	-	Sparringa and Owens (1999)
Aspergillus sp.	-	-	24.10	-	-	Carter *et al.* (2004)
Monascus pilosus	-	0.26	-	-	13.20	Yu *et al.* (2005)
Aspergillus sp. BCRC 31742	18.50	3.43	185.00	20.40	137.00	Hsieh *et al.* (2007)
Monascus pilosus BCRC31527	17.70	0.72	40.40	4.28	35.90	Hsieh *et al.* (2007)
Rhizopus oligosporus BCRC 31996	2.09	0.40	188.00	2.34	13.20	Hsieh *et al.* (2007)
Rhizopus oryzae ATCC 20344	-	-	160.00	-	-	Liao *et al.* (2008)
Aspergillus sp. BCRC 31742	21.56	5.48	250.00	32.60	160.00	This study

Table 7: Production of GlcN using wild type fungi cultivated in fermenter

DO value	Biomass (g L^{-1})	GlcN conc. (g L^{-1})	Content (g g^{-1} -biomass)	Productivity (mg L^{-1} h)	Yield (g g^{-1} -carbon source)
Uncontrolled	14.2	1.07	0.08	6.00	0.04
5%	13.7	1.25	0.09	7.44	0.05
10%	14.6	3.91	0.27	23.30	0.16
15%	11.1	3.34	0.30	13.80	0.13
20%	15.2	3.51	0.23	20.90	0.14
40%	15.5	3.18	0.21	18.90	0.13
60%	13.0	2.46	0.19	14.60	0.10

the GlcN concentration resulted was 1.07 g L^{-1}; biomass conc. 14.2 g L^{-1}; content 0.75 g g^{-1} biomass; yield 0.04 g g^{-1} carbon source; productivity 6.00 mg L^{-1} h. The results were worse than that in flask culture mentioned above.

In the previous study, it was found that agitation affected the growth of fungi which means in fermenter, Dissolved Oxygen (DO) plays important role. Thus in this section, fermenter cultivation was only focused in variation of DO value. In order to increase the glucosamine concentration, the increase flux of oxygen was needed. If the dissolved oxygen was at range 10-20%, GlcN concentration was high. When DO was 10%, GlcN concentration achieved was 3.91 g L^{-1}; biomass conc. 14.6 g L^{-1}; content 0.27 g g^{-1} biomass; yield 0.16 g g^{-1} carbon source; productivity 23.3 mg L^{-1} h. If the dissolved oxygen was higher than 40%, besides it was difficult to control DO value, it also made pellets become hardly to bind oxygen thus resulted in poor biomass concentration or even GlcN concentration. Therefore, DO value which was <40% is better conclusively. Overall data can be shown in Table 7.

CONCLUSION

Higher GlcN production has been investigated using wild type fungi, *Aspergillus* sp. BCRC 31742. The cultivation was carried out in a shake flask, incubated at 30°C, shaken at 200 rpm, initial pH of medium 7.0 and with fermentation time 7 days. An optimized fermentation medium has been resulted by employing response surface methodology with 2 main factors such as WF sugar and peptone. By using optimum medium constituents, the biomass concentration could up to 21. 6 g L^{-1} with GlcN concentration of 5.48 g L^{-1}. There is now a considerable body of literature describing the use of statistical design methods for process optimization at small scale. This finding will be also applied to enhance the glucosamine production at larger scale in the future.

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REFERENCES

Bowman, S.M. and S.J. Free, 2006. The structure and synthesis of the fungal cell wall. Bio Essays, 28: 799-808.

Box, G.E.P. and N.R. Draper, 1987. Empirical Model-Building and Response Surfaces. John Wiley and Sons, Inc, New York, ISBN-13: 9780471810339, pp: 699.

Braun, S. and S.E. Vecht-Lifshitz, 1991. Mycelial morphology and metabolite production. Trends Biotechnol., 9: 63-68.

Cao, L., Y. Jiang, Y. Yu, X. Wei and W. Li, 2008. Methods for producing glucosamine from microbial biomass. US Patent 0188649 A1.

Carter, S.B., S.E. Nokes and C.L. Crofcheck, 2004. The influence of environmental temperature and substrate initial moisture content on *Aspergillus niger* growth and phytase production in solid-state cultivation. Trans. Am. Soc. Agric. Eng., 47: 945-949.

Chatterjee, S., M. Adhya, A.K. Guha and B.P. Chatterjee, 2005. Chitosan from *Mucor rouxii*, production and physico-chemical characterization. Proc. Biochem., 40: 395-400.

Deng, M.D., D.K. Severson, A.D. Grund, S.L. Wassink and R.P. Burlingame *et al.*, 2005. Metabolic engineering of *Escherichia coli* for industrial production of glucosamine and *N-acetylglucosamine*. Metab. Eng., 7: 201-214.

D'Ambrosio, E., B. Casa, R. Bompani, G. Scali and M. Scali, 1981. Glucosamine sulphate: A controlled clinical investigation in arthrosis. Pharmacotherpeutica, 2: 504-508.

Ferrer, J., G. Paez, Z. Marmol, E. Ramones, H. Garcia and C.F. Forster, 1996. Acid hydrolysis of shrimp-shell wastes and the production of single cell protein from the hydrolysate. Bioresour. Technol., 57: 55-60.

Gbolagade, J., A. Sobowale and D. Adejoye, 2006. Optimization of sub-merged culture conditions for biomass production in *Pleurotus florida* (mont.) Singer, a Nigerian edible fungus. Afr. J. Biotechnol., 5: 1464-1469.

Hajji, M., A. Rebai, N. Gharsallah and M. Nasri, 2008. Optimization of alkaline protease production by *Aspergillus clavatus* ES1 in *Mirabilis jalapa* tuber powder using statistical experimental design. Applied Microbiol. Biotechnol., 79: 915-923.

Hsieh, J.W., H.S. Wu, Y.H. Wei and S.S. Wang, 2007. Determination and kinetics of producing glucosamine using fungi. Biotechnol. Progress, 23: 1009-1016.

Jung, W.J., G.H. Jo, J.H. Kuk, K.Y. Kim and R.D. Park, 2005. Demineralization of crab shells by chemical and biological treatments. Biotechnol. Bioprocess Eng., 10: 67-72.

Kennedy, M. and D. Krouse, 1999. Strategies for improving fermentation medium performance: A review. J. Ind. Microbiol. Biotechnol., 23: 456-475.

Kim, W.J., W.G. Lee, K. Theodore and H.N. Chang, 2001. Optimization of culture conditions and continuous production of chitosan by the fungi, *Absidia coerulea*. Biotechnol. Bioprocess Eng., 6: 6-10.

Kuk, J.H., W.J. Jung, G.H. Jo, Y.C. Kim, K.Y. Kim and R.D. Park, 2005. Production of N-acetyl-β-D-glucosamine from chitin by *Aeromonas* sp. GJ-18 crude enzyme. Applied Microb. Biotechnol., 68: 384-389.

Li, Z.J., V. Shukla, K.S. Wenger, A.P. Fordyce, A.G. Pedersen and M.R. Marten, 2002. Effects of increased impeller power in a production-scale *Aspergillusoryzae* fermentation. Biotechnol. Progress, 18: 437-444.

Liao, W., Y. Liu, C. Frear and S. Chen, 2008. Co-production of fumaric acid and chitin from a nitrogen-rich lignocellulosic material-dairy manure-using a pelletized filamentous fungus *Rhizopus oryzae* ATCC 20344. Bioresour. Technol., 99: 5859-5866.

Lim, J.M., S.W. Kim, H.J. Hwang, J.H. Joo, H.O. Kim, J.W. Choi and J.W. Yun, 2004. Optimization of medium by orthogonal matrix method for submerged mycelial culture and exopolysaccharide production in *Collybia maculata*. Applied Biochem. Biotechnol., 119: 159-170.

Mattia, A., 2004. Agency response letter GRAS notice No. GRN 000150. CFSAN/Office of Food Additive Safety.

Montgomery, D.C., 1991. Design and Analysis of Experiments. John Wiley and Sons Inc., New York.

Niladevi, K.N., R.K. Sukumaran, N. Jacob, G.S. Anisha and P. Prema, 2009. Optimization of laccase production from a novel strain-*Streptomyces psammoticus* using response surface methodology. Microbiol. Res., 164: 105-113.

Nwe, N., S. Chandrkrachang, W.F. Stevens, T. Maw, T.K. Tan, E. Khor and S.M. Wong, 2002. Production of fungal chitosan by solid state and submerged fermentation. Carbohydrate Polymers, 49: 235-237.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelia processes. Biotech. Adv., 22: 189-259.

Ratnam, B.V.V., S.S. Rao, D.R. Mendu, M.N. Rao and C. Ayyanna, 2005. Optimization of medium constituents and fermentation conditions for the production of ethanol from palmyra jaggery using response surface methodology. World J. Microbiol. Biotechnol., 21: 399-404.

Rattanakit, N., S. Yang, M. Wakayama, A. Plikomol and T. Tachiki, 2003. Saccharification of chitin using solid-state culture of *Aspergillus* sp. S1-13 with shellfish waste as a substrate. J. Biosci. Bioeng., 95: 391-396.

Rogers, B.D., 2004. Notification of GRAS determination for REGENASURETM glucosamine hydrochloride. http://www.accessdata.fda.gov/scripts/fcn/gras_notices/400760A.PDF.

Ruiz-Teran, F. and J.D. Owens, 1996. Chemical and enzymic changes during the fermentation of bacteria-free soya bean tempe. J. Sci. Food Agric., 71: 523-530.

Sashiwa, H., S. Fujishima, N. Yamano, N. Kawasaki and A. Nakayama *et al*., 2002. Production of N-acetyl-D-glucosamine from á-chitin by crude enzymes from *Aeromonas hydrophila* H-2330. Carbohydr. Res., 337: 761-763.

Sitanggang, A.B., H.S. Wu and S.S. Wang, 2009. Determination of fungal glucosamine using HPLC with 1-Napthyl isothiocyanate derivatization and microwave heating. Biotechnol. Bioprocess Eng., 14: 819-827.

Sparringa R.A. and J.D.I. Owens, 1999. Glucosamine content of tempe mould, *Rhizopus oligosporus*. Int. J. Food Microbiol., 47: 153-157.

Xiong, C., C. Shouwen, S. Ming and Y. Ziniu, 2005. Medium optimization by response surface methodology for poly-ã-glutamic acid production using dairy manure as the basis of a solid substrate. Applied Microbiol. Biotechnol., 69: 390-396.

Yu, K.W., Y.S. Kim, K.S. Shin, J.M. Kim and H.J. Suh, 2005. Macrophage-stimulating activity of exo-biopolymer from cultured rice bran with *Monascus pilosus*. Applied Biochem. Biotechnol., 126: 35-48.