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Reutilization of waste fungal biomass for concomitant production of proteo-chitinolytic enzymes and their catalytic products by *Alcaligenes faecalis* SK10

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Fungal biomass, being organic waste, could be an excellent source of protein, carbohydrate and minerals. However, it has not been exploited fully until now. Efficient management of this waste can not only address the environmental impact on its disposal but also yield value-added metabolites. In the present study, in order to explore its potential, we subjected dead fungal biomass of *Aspergillus niger* SKN1 as substrate for both fermentative and enzymatic biodegradation, respectively by potent proteo-chitinolytic bacteria *Alcaligenes faecalis* SK10 and its enzyme cocktail. The results revealed that reasonable amount of protease and chitinase could be biosynthesized by the fermentative mode of utilization, while a mixture of amino acid, peptides and low-molecular weight amino-sugar (mono and oligomeric form of N-acetylglucosamine) could be generated through enzymatic hydrolysis. The physicochemical condition of both the bioprocess was subsequently optimized through statistical approach. The projected utilization of waste zero-valued fungal biomass offer a sustainable and environmentally sound method for production of microbial metabolites and large scale execution of the same could be proficient and in tune with the principle of circular economy.

Keywords: N-acetylglucosamine, Chitinase, Fungal biomass, Hydrolysis, Protease, Waste valorization

Fungi are known to adapt to severe environmental constraints which endorses their use in bioprocessing industries for the production of a large variety of bioactive compound like extracellular and intracellular enzymes, organic acids, antibiotics, ethanol, etc., and thereby find extensive application in fermentation industries. As example, the prototypical fungus *Aspergillus* is recognized as the best enzyme producer which is widely used in the enzyme producing industries across the world[1-2]. Industrial production of value-added bioactive compounds are increasing day by day with the help of modern biotechnology and sustainable fungal species, but at the same time, large amounts of fungal cell biomass was generated as by-product during downstream processing which are often discarded as waste. An average sized bioactive compounds production facility produces several thousands of tons of cell biomass per year which have no suitable utilization outlet. Nonetheless, fungal cell biomass is a potential source of carbohydrates, glycoproteins, minerals, etc., and therefore, reutilization of the same could be promising in purview of circular economy.

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Chitin is most plenteous amino-polysaccharides composed of repetitive unit of N-acetyl glucosamine (GlcNAc), present in fungal cell wall and contributes up to 10 to 20% of its dry weight[3]. Besides, fungal cell wall also contains alkali insoluble β-1-6 glucan, β-1-3 glucan, mannoproteins, O and N linked mannans, glycoproteins and melanin[4]. Fungal cell wall carbohydrates are basically glucose, galactose, mannose, N-acetylglucosamine, and N-acety-lgalactosamine which is present in the cell was in approximate ratio of 22:11:8:2:1, respectively[5]. However, the ratio of fungal cell wall carbohydrates and proteins is varied depending on fungal nutrition source and also on the fungal strain type.

Chitin and its digested products could be obtained from fungal cell wall and crustacean shell has many applications, mainly in the food and pharmaceutical sectors, due to their cationic, biocompatible, antimicrobial and antioxidant properties[6,7]. Consumption of N-acetylglucosamine positively affects the human health by combating against various immune system related diseases (e.g. osteoarthritis) along with other therapeutic advantageous (e.g. protect the mucosal lining of stomach and intestine, preventing cancer and skin-related complications, etc.) and therefore served

as potential food additives.[8-11]. Though industries are mainly relies on the crustacean shell for production of such metabolites, it has several drawbacks such as unequal availability of shell due to seasonal and geographical alteration, their unpredictable composition and physicochemical properties, and use of aggressive chemicals in the extraction process collectively enforce great challenges. Chemical extraction processes such metabolites from crustacean shell requires higher energy input and the use of strong chemicals, which have several environmental consequences[12]. On the contrary, after the downstream processing, industries generally throw away the post-harvested fungal biomass which could be used as an alternative source for production of such valuable products in controlled conditions by simpler extraction process and using less-aggressive chemicals[13]. Moreover, the availability of the fungal biomass will not be an issue.

Chitin degrading enzyme, chitinase of microbial origin has immense industrial application like in the production of single cell protein, control of plant pathogens (fungi and insects), fungal protoplast preparation, and preparation of pharmaceutical products such as chitosan, chito-oligosaccharides, etc.[14-18]. Chitinase is also used in the production of GlcNAc from chitin-containing waste through enzymatic degradation which is considered as a sustainable and economic approach[19,20]. Likewise, microbial protease becomes an indispensable enzyme that has the ability to produce bioactive peptides and amino acids from proteins. Proteases have application in many industrial fields like medical, detergent, brewing, and meat processing as well as in basic biotechnological research while their hydrolyzed products have potential enrolment in food and feed sector [21-23].

In our previous study, it was reported that proteo-chitinolytic microorganism can produce both the enzymes chitinase and protease from crustacean wastes as substrate and reasonable amount of amino sugar and free amino acids are also get accumulated in the medium during the process [24]. In this context, here, we have made an attempt to valorize waste biomass of one of the most commonly explored fungi *Aspergillus niger* for production of chitinase and protease. The crude enzyme preparation was further used for enzymatic production of chitin and protein hydrolyzed value-added products from the fungal biomass.

Materials & Method

Microorganisms

Both the microbes used in the present study were previously isolated and explored. The proteo-chitinolytic bacteria *Alcaligenes faecalis* SK10 (GenBank Accession no. MT628354) was isolated from marine environment and lingo-cellulolytic fungus *Aspergillus niger* SKN1 (GenBank Accession no. MW479135) was isolated from the nearest forest area [24-25]. The bacteria was maintained on colloidal chitin agar (CCA) slants at 4ºC, while at the same temperature the fungus was preserved on potato dextrose agar (PDA) slants. Prior experiment, both the microbes were freshly grown a by culturing them in their respective selective medium according to Pal *et al*.[24] and Mondal *et al*.[25].

Preparation of dead powdered fungal biomass and colloidal chitin

In our previous study lingo-cellulolytic enzyme production was carried out by *A. niger* SKN1 through submerged fermentation[25]. After separation of crude enzyme cocktail the fungal biomass was harvested that was used in the present study. Before use, the biomass was vigorously washed, autoclaved and subsequently oven dried. The dried fungal biomass was subsequently converted into powder using mortar and pestle.

Production of colloidal chitin was carried out as per the method adopted by Pal *et al*.[24]. The process started with vigorous mixing of flake chitin in ice-cold hydrochloric acid (11.65 N) in 1:20 ratio and left for 24 h of under shaking (at 150 rpm). The suspension was poured in cold ethanol (50%, v/v) followed by vigorous shaking for 2 h and subjected to overnight incubation at 20ºC under static condition. After centrifugation (10,000 rpm for 10 min), the precipitate was collected and suspended in water. The pH of the colloidal chitin was brought to neutral by repeated serial centrifugation, resuspended, and washing with sterile distilled water.

Optimization of fermentative production enzymes and metabolites

Submerged fermentation (SmF) was executed in 250 mL of Erlenmeyer flask containing 50 mL of previously standardized medium salt in which dead powdered fungal biomass was added as economical substrate. The medium was inoculated with freshly grown *A. faecalis* SK10 culture (~5×10^6cfu/mL) and incubated at 120 rpm. To maximize the proteo-chitinolytic enzymes production, the impact of

fermentation time, medium pH, incubation temperature, level of phosphate (equal amount of K_2HPO_4 and KH_2PO_4) and yeast extract (as additional nitrogen source) were assessed by response surface methodology (RSM) using Box-Behnken design (BBD) by changing the medium pH from 5.0 to 9.0, phosphate source from 0.15 to 0.25 (%), and nitrogen source from 0.1 to 0.3 (%) in the medium, incubation period from 48 to 96 h, and temperature from 30 to 40ºC following the method of Halder *et al.*[26]. These independent variables were tested in three equi-distance levels [low (-1), intermediate (0), and high (+1)] through 46 experimental runs as per BBD. During the course of fermentation, the liquid medium was withdrawn at a regular interval of 24 h and centrifuged (5000 rpm for 10 min at 4°C) to collect supernatant. The chitinase, protease, amino sugar, free amino acid and soluble peptide level in the supernatant was measured.

Optimization of enzymatic hydrolysis of fungal biomass

To determine the proficiency of produced proteo-chitinolytic enzymatic cocktail and in order to get both the chitin and protein hydrolyzed products from fungal biomass, the same was further subjected for the crude enzymatic treatment. Ten milliliter of reaction mixture containing 1 gram of fungal biomass, buffer and enzyme was incubated at under vigorous shaking. As like previous, BBD model based RSM was adopted to attain the optimized condition for production of amino sugar, free amino acid and soluble peptide by analyzing the effect of four process variables viz. incubation period (60-180 min), incubation temperature (30-40ºC), pH (5-9), enzymatic dose (volume) (0.5-1.5 mL) in 29 experimental run.

Enzymatic assay for chitinase and protease

To determine chitinase activity, 0.1 mL of the culture supernatant was added to 1 mL of 1.0% (w/v) colloidal chitin (in 0.1Mphosphate buffer, pH 7.0) and incubated at 35ºC for 60 min under vigorous shaking. The generated reducing sugar content was estimated according to Halder *et al.*[26] by taking *N*-acetylglucosamine as a reference compound. Chitinase activity was expressed in unit (U) which is defined as the amount of enzyme needed to release 1 µmol of *N*-acetylglucosamine equivalent per minute under standard experimental conditions.

To determine protease activity, culture supernatant (50 µL) was mixed with 450 µL 0.2 M phosphate buffer (pH 7.0) containing 1% (w/v) casein and incubated for 1 h at 35ºC. The reaction was terminated by adding 10% trichloroacetic acid followed by incubation at ambient temperature for 15 min. After centrifugation (10,000 rpm for 10 min), the free amino acids and soluble peptides content in the supernatant was measured according to Rakshit *et al.*[27] by taking tyrosine as reference. Protease activity was expressed in unit which was defined as the amount of enzyme required to liberate 1 µmol of tyrosine (equivalent) per min under standard assay conditions.

Estimation of amino sugars, and free amino acids and soluble peptides

The amino sugar content in the culture supernatant and enzymatic hydrolysate was estimated according to Reissig *et al.*[28]. An aliquot 0.5 mL of sample was mixed with 100 microliter of potassium tetraborate solution and heated in a boiling water bath for 3 min. After cooling, 3 mL of dimethylaminobenzaldehyde (DMAB) reagent was added to the mixture and incubated for 20 min at 36ºC in a water bath. The absorbance was taken 544 nm with *N*-acetylglucosamine as reference. The free amino acids and soluble small peptides were estimated according to Lowry *et al.*[29] taking tyrosine as reference.

Chromatographic analysis of amino sugars and free amino acid

The amino acids were also qualitatively analyzed on pre-coated (0.25 mm) silica gel thin layer chromatography (TLC) plates (Merck, Germany), using 3:1:1 (v/v/v) n-butanol/ acetic acid/water as mobile phase. The amino acids were visualized by spraying with ethanolic solution of ninhydrin.

The presence and quantity of amino sugars generated during fermentation and enzymatic hydrolysis of fungal biomass was analyzed through HPLC. Both samples were diluted with 50 mM TrisHCl (pH 8.8) at the ratio of 1:3 (W/V) and kept for 1 h at 4ºC followed by centrifugation at 10,000 rpm for 20 min. The collected supernatant was filtered (0.22 mm pore size filter) and then used for HPLC analysis (Agilent Technology, 1200 infinity series, USA) according to Halder *et al.*[30] using carbohydrate-NH_2 column, acetonitrile: water (70:30) mobile phase and at 1 mL/min flow rate. The products were analyzed by UV detector at 210 nm.

Scanning electron microscopy (SEM)

Fermented and enzymes treated fungal biomass were fixed with 10% glutaraldehyde and dehydrated with a gradient of ethanol and hexamethyldisilazane

(HMDS). The samples were then gold coated and observed under SEM (Hitachi-530, Japan) according to Halder *et al*.[30].

Antioxidative assay

Assay of 2,2-diphenyl-1-picrylhydrazyl DPPH radicalscavenging activity

The culture supernatant (150 µl), and enzymatic hydrolyzed supernatant (150 µl) was separately mixed with 37.5 µl of methanolic DPPH radical solution (0.75 mM). After vigorously shaking, both the mixture was incubated for 20 minutes in dark place at ambient temperature and the absorbance was measured at 517 nm by taking DPPH solution without any supernatant as control[26]. The antioxidant compounds were also visualized on TLC plates by spraying with the same DPPH solution (0.75 mM), followed by heating.

DPPH-Radical scavenging sctivity (RSA) % = $[(A517_{control} - A517_{sample}) / A517_{control}] \times 100$

Assay of 2,2'-azino-bis (3 ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical-scavenging activity

ABTS radical-scavenging activity was measured, following the method of Halder *et al.*[26] with minor modification. A mixture of 50 mL of 2 mM ABTS and 200 µl of 70mM potassium persulfate was incubated for 24 h for the generation of stable ABTS radicals. The working solution was prepared by diluting the stock solution with methanol to reach an absorbance of 0.7 ± 0.02 at 734 nm ($A_{control}$). One millilitre of working solution of ABTS radical was mixed with the culture supernatant (150 µl) and enzymatic hydrolyzed supernatant (150 µl) separately, and the absorbance (A_{sample}) was noted after 10 min for each sample. The radical-scavenging activities in both cases were calculated as follows:

ABTS-Radical scavenging sctivity (RSA) % = $[(A_{control} - A_{sample}) / A_{control})] \times 100$

Statistical analysis

All the experiments were carried out in triplicates and results were represented as data represented as their mean±SE. RSM optimization was carried out using Design Expert 8.0.4 (Stat-Ease, Inc., Minneapolis, USA) statistical software package.

Results and Discussion

Utilization of fungal biomass for enzyme production

From ancient time fungi are being employed for manufacturing of foods and beverages like alcohol and bread. In the present age, different molds and yeasts in their wild and genetically engineered form are used in the industrial scale production of broad spectrum of compounds like enzymes, organic acids, vitamins, colorants, antibiotics, lipids, polysaccharides, etc. as fungi may improve various attributes of the product of interest[31]. Filamentous fungi are considered as ideal sources for production of different enzymes, and at present more than half of the industrial enzymes are of fungal origin which has diversified enrollment in food, feed, textile, pharmaceutical, pulp and paper, detergent, and therapeutic sectors. Among the fungal genera, *Aspergillus* is the most exploited one, employed for the synthesis of citric acid, protease, phytase, L-asparaginase, glycosyl hydrolases, keratinase, pectinase, etc. which have diverse applications[32]. In industrial production of any fungal biosynthetic product, huge amount of fungal biomass is produced as principal byproduct during downstream processing which have no proper utilization outlet, and therefore poorly valorized[33]. Being organic origin, fungal biomass could be considered as an excellent source of protein, carbohydrate and minerals, but its underutilization as well as the environmental impact with respect to its disposal have been a matter of concern. If treated efficiently this waste fungal biomass can serve as substrate for biosynthesis of microbial enzymes.

The dead fungal mycelial biomass principally contains cell wall materials such as polysaccharides, lipids and proteins. In polysaccharide fraction, chitin is predominant; however, its relative concentration is varied species to species. It was reported that, the amount of chitin and protein is relatively highin fungal cell walls[34]. In the present study, biomass of *Aspergillus niger* SKN1 (which was recovered after lignocellulolytic enzyme production in our earlier study[25]) was subjected for production of chitinase and protease by potent proteo-chitinolytic strain *Alcaligenes faecalis* SK10 which was explored in our previous study[24]. The utilization of dead fungal biomass for the enzyme production was carried out through submerged fermentation and the effect of five physico-chemical factors viz. fermentation time, incubation temperature, medium pH, concentration of phosphate and concentration of yeast extract were investigated through BBD design of RSM to evaluate their effect and to point out the optimum conditions for the fermentation in order to achieve consistent products. The combinatorial effects of all the factors for chitinase and protease production are represented in Table 1. The results suggest that the proposed

Table 1 — BBD design with experimental and predicted response for chitinase and protease production utilizing fungal biomass

Run no	A: Fermentation time (h)	B: Incubation temp. (^oC)	C: Medium pH	D: Phosphate (%)	E: Yeast extract (%)	Y1: Chitinase (U/mL)		Y2: Protease (U/mL)	
						Experimental	Predicted	Experimental	Predicted
1	72	35	5	0.2	0.1	30.76	30.78	26.76	26.69
2	48	35	7	0.25	0.2	23.56	23.7	20.43	20.5
3	72	40	5	0.2	0.2	30.45	30.48	25.67	25.17
4	72	30	7	0.2	0.1	33.67	32.95	26.14	25.7
5	72	35	5	0.25	0.2	32.43	32.07	26.76	26.72
6	72	30	5	0.2	0.2	30.21	30.27	26.19	25.79
7	72	30	7	0.15	0.2	30.65	30.7	26.76	27.04
8	72	40	7	0.15	0.2	31.67	31.85	26.56	26.88
9	48	35	7	0.2	0.1	23.45	24.04	19.34	19.58
10	72	35	7	0.25	0.1	31.42	31.89	25.21	25.45
11	96	35	7	0.2	0.1	26.17	26.25	23.11	23.46
12	48	35	7	0.15	0.2	23.98	23.42	21.87	21.7
13	72	35	7	0.2	0.2	34.14	34.14	28.37	28.29
14	72	35	9	0.25	0.2	31.87	31.46	24.66	24.57
15	72	40	9	0.2	0.2	30.5	30.91	25.1	25.4
16	72	35	7	0.25	0.3	33.21	33.72	27.15	27.4
17	48	35	9	0.2	0.2	23.65	23.44	20.04	19.58
18	96	35	5	0.2	0.2	24.21	24.57	22.12	22.56
19	72	35	9	0.2	0.1	32.89	32.45	22.65	22.79
20	96	35	7	0.15	0.2	25.41	24.73	23.76	23.64
21	48	35	5	0.2	0.2	22.12	21.79	19.32	19.81
22	96	35	7	0.25	0.2	26.87	26.88	24.23	24.35
23	72	30	7	0.25	0.2	33.78	33.54	27.23	26.93
24	72	30	7	0.2	0.3	31.76	31.47	26.87	27.03
25	72	30	9	0.2	0.2	31.62	32.07	24.98	25.38
26	72	35	7	0.15	0.1	33.1	33.24	27.1	26.83
27	72	35	9	0.2	0.3	31.78	31.17	27.26	27.41
28	72	40	7	0.2	0.3	31.45	31.74	25.81	26.22
29	72	35	5	0.2	0.3	30.76	30.6	23.75	23.69
30	96	30	7	0.2	0.2	25.45	25.76	23.65	23.75
31	48	30	7	0.2	0.2	23.26	23.63	20.78	20.98
32	72	35	7	0.2	0.2	34.43	34.14	28.31	28.29
33	72	35	7	0.2	0.2	33.56	34.14	27.87	28.29
34	72	35	7	0.2	0.2	34.19	34.14	28.37	28.29
35	72	35	9	0.15	0.2	31.65	31.97	26.78	26.87
36	96	40	7	0.2	0.2	25.76	25.4	23.67	23.58
37	96	35	9	0.2	0.2	24.67	25.15	23.13	22.62
38	72	35	7	0.2	0.2	34.21	34.14	28.43	28.29
39	72	35	5	0.15	0.2	28.76	29.13	24.76	24.9
40	72	40	7	0.2	0.1	31.87	31.73	26.1	25.92
41	72	35	7	0.2	0.2	34.31	34.14	28.4	28.29
42	72	40	7	0.25	0.2	31.54	31.43	26.76	26.5
43	48	40	7	0.2	0.2	23.34	23.04	20.54	20.55
44	48	35	7	0.2	0.3	22.98	23.27	21.76	21.38
45	96	35	7	0.2	0.3	25.76	25.55	23.56	23.29
46	72	35	7	0.15	0.3	29.76	29.94	26.76	26.5

quadratic model is suitable to demonstrate both the enzyme production. Statistical analysis reveal the significance of the model with a computed F-value of 158.10 and 112.76, respectively for chitinase and protease production by *A. faecalis* SK10 production. The result was endorsed by the model P-value (Prob> F) lower than 0.0001 (Table 2) which implied that only 0.01% chance that an F-value this large could occur due to noise. The lack of fit F-value of 2.79 and 3.61 for chitinase and protease production implied that the same is not significant for both the cases. There is 12.96 and 7.97% chance that the Lack of Fit F-value was occurred due to noise. The statistical indices like coefficient of variation (CV) and coefficient of determination (R^2) were taken under consideration to judge the competence of the experimental model of RSM[35]. The CV value is a relative measure of dispersion, obtained by expressing

Table 2 — ANOVA and other statistical indices for chitinase production optimization through BBD

Source	Sum of Square	df	Mean Square	F-value
Model	701.65	20	35.08	158.1
A	20.16	1	20.16	90.85
B	0.912	1	0.912	4.11
C	4.98	1	4.98	22.46
D	5.88	1	5.88	26.5
E	2.15	1	2.15	9.7
AB	0.0132	1	0.0132	0.0596
AC	0.2862	1	0.2862	1.29
AD	0.8836	1	0.8836	3.98
AE	0.0009	1	0.0009	0.0041
BC	0.4624	1	0.4624	2.08
BD	2.66	1	2.66	11.97
BE	0.555	1	0.555	2.5
CD	2.98	1	2.98	13.41
CE	0.308	1	0.308	1.39
DE	6.58	1	6.58	29.65
A^2	621.31	1	621.31	2799.85
B^2	13.47	1	13.47	60.72
C^2	33.64	1	33.64	151.6
D^2	9.07	1	9.07	40.85
E^2	7.47	1	7.47	33.65
Residual	5.55	25	0.2219	
Lack of Fit	5.09	20	0.2545	2.79
Pure Error	0.4568	5	0.0914	
Cor Total	707.2	45		

Model R^2 = 0.9922, Adjusted R^2 = 0.9859, Predicted R^2 = 0.9703, Adequate precision = 38.7984, PModel <0.0001, PLack of Fit = 0.1296, CV = 1.6%

$Y_{Chitinase}$ =+34.14+1.12A-0.2387B+0.5581C+0.6063D-0.3669E+0.0575 AB-0.2675AC+0.4700AD+0.0150AE-0.3400BC-0.8150BD+0.3725BE -0.8625CD-0.2775CE+1.28DE-8.44A^2-1.24B^2-1.96C^2-1.02D^2- BBD 0.9250E^2

Table 3 — ANOVA and other statistical indices for protease production optimization through BBD

Source	Sum of Square	df	Mean Square	F-value
Model	308.22	20	15.41	112.76
A	33.5	1	33.5	245.08
B	0.357	1	0.357	2.61
C	0.0333	1	0.0333	0.2437
D	0.2304	1	0.2304	1.69
E	2.65	1	2.65	19.38
AB	0.0169	1	0.0169	0.1237
AC	0.021	1	0.021	0.1538
AD	0.912	1	0.912	6.67
AE	0.9702	1	0.9702	7.1
BC	0.1024	1	0.1024	0.7493
BD	0.0182	1	0.0182	0.1334
BE	0.2601	1	0.2601	1.9
CD	4.24	1	4.24	31.05
CE	14.52	1	14.52	106.21
DE	1.3	1	1.3	9.51
A^2	234.55	1	234.55	1716.19
B^2	6.95	1	6.95	50.87
C^2	33.73	1	33.73	246.78
D^2	2.75	1	2.75	20.14
E^2	12.2	1	12.2	89.29
Residual	3.42	25	0.1367	
Lack of Fit	3.2	20	0.1598	3.61
Pure Error	0.2213	5	0.0443	
Cor Total	311.63	45		

Model R^2 = 0.989, Adjusted R^2 = 0.9803, Predicted R^2 = 0.958, Adequate precision = 34.8901, P_{Model} <0.0001, $P_{Lack\ of\ Fit}$ = 0.0797, CV = 1.49%

$Y_{Protease}$ = +28.29+1.45A-0.1494B-0.0456C-0.1200D+0.4069E+0.0650 AB+0.0725AC+0.4775AD-0.4925AE+0.1600BC-0.0675BD-0.2550BE-1.03CD+1.91CE+0.5700DE-5.18A^2-0.8925B^2-1.97C^2-0.5617D^2-1.18E^2

the standard deviation as a percentage of mean, and a model can be considered reasonably reproducible if the CV is not greater than 10%. In the present case, the CV values for chitinase and protease were 1.6 and 1.49, which illustrated that the model can be considered reasonably reproducible. The predicted R^2 values (0.9703 and 0.9580) were in reasonable agreement with the adjusted R^2 (0.9859 and 0.9803) as the differences between both the R^2 are less than 0.2. The regression equations of the model for both the enzymes are represented in Tables 2 and 3.

Adequate precision (AP) is another parameter that measures the signal to noise ratio and the value greater than 4 is anticipated. The calculated AP value of 38.798 (for chitinase) and 34.890 (for protease) endorsedan adequate signal. All the statistical indices suggested that the model was pertinent to represent the real relationship among the selected factors for proteo-chitinolytic enzyme production by *A. faecalis* SK10 utilizing fungal biomass. In RSM-based optimization, response surface plots narrate visual interpretation of the interaction between two factors (with the other variables at their central value) and facilitate to locate optimum experimental conditions[35-36]. The topmost significant interactions for chitinase and protease production were represented in Fig. 1. Using numerical optimization tool, solutions with higher desirability suggested that the maximum production of chitinase and protease production could be achieved when fermentation time of 74.5 h, Incubation temperature of 34.3°C, medium pH of 7.05, phosphate source of 0.2128% and yeast extract of 0.2138% provided. To verify the prediction, validation experiment was carried out providing the aforesaid conditions which was in good agreement with predicted result. In the culture supernatant 14 and 9 µg/mL of amino sugars, and amino acids with peptide was accumulated at the time of harvesting (74.5 h), and at that time 5.63 and 6.84 µg/mL of ascorbic acid equivalent ABTS and DPPH radical scavenging activity were estimated, respectively. The degradation of the fungal biomass/spore during the

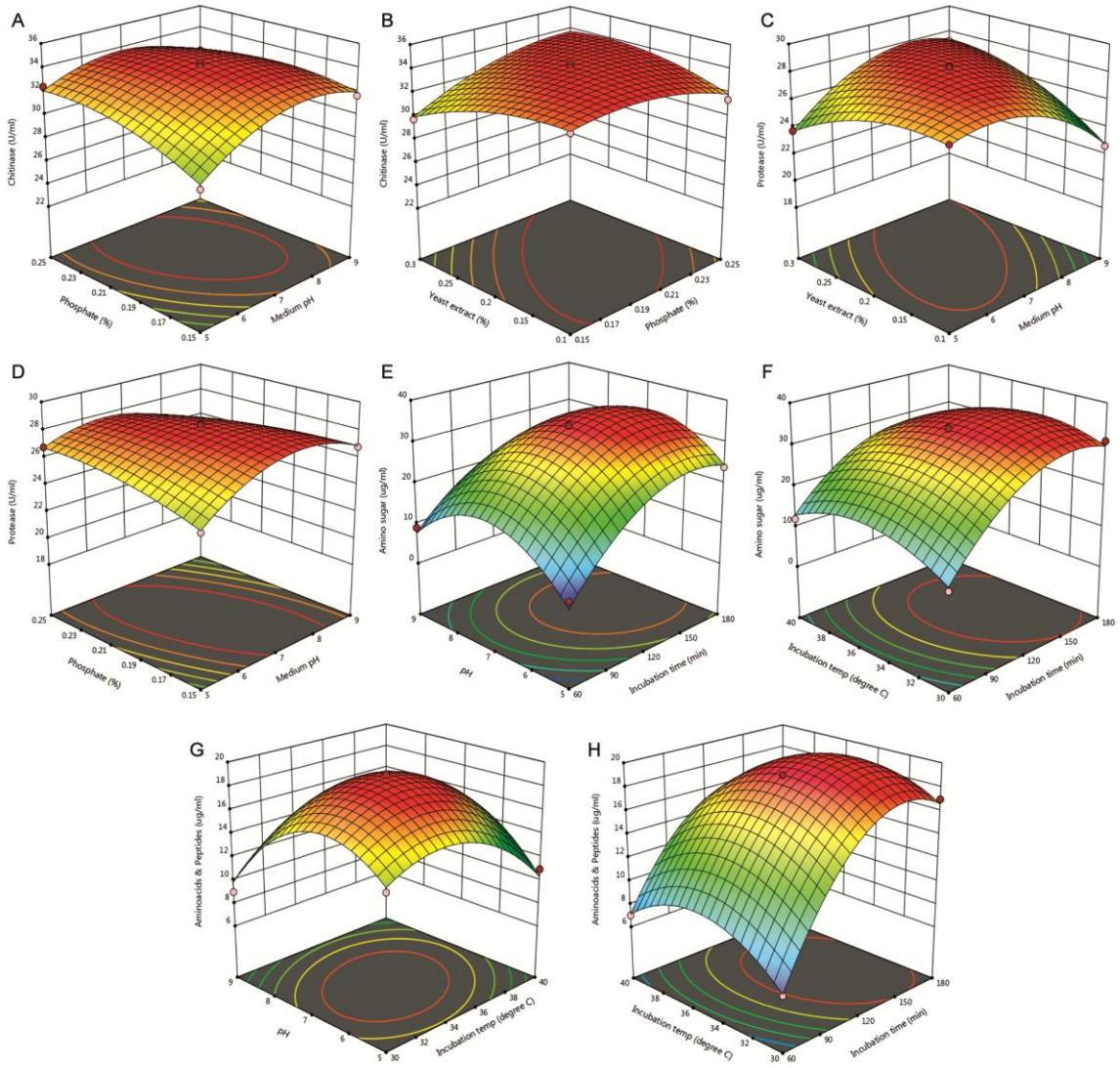

Fig. 1 — Response surface and contour plots of the most effective interactions among the variables on chitinase (A, B), protease (C, D) production by the bacterial isolate, and amino sugar (E, F), aminoacid & peptide (G, H) generation by the enzyme cocktail

course of fermentation was visually verified by SEM analysis (Fig. 2). It is apparent that the fungal spore/mycelium was degraded by the action of microbial enzymes that was secreted by *A. faecalis* SK10 during the course of fermentation. Microbial production of chitinase by utilizing mycelial mass of *Aspergillus niger* and *Sclerotium rolfsii* were reported by Tagawa & Okazaki[37] and Vyas & Deshpande[38]. Narayana & Vijayalakshmi[39] documented chitinase production efficiency of *Streptomyces* sp. ANU 6277 in the fermentation medium supplemented with fungal mat of *Fusarium oxysporum* and *Penicillium citrinum* separately. The growth and chitinase production of *Streptomyces halstedii* AJ-7 growth was greatly enhanced at submerged condition in the medium containing colloidal chitin supplemented with cell wall preparation of *Fusarium oxysporium*[40]. Our result is in agreement with the said reports; however, comparatively higher amount of enzyme production was noticed in the present study. Fungal biomass is not an excellent source of chitin and protein in terms of quantity and therefore could not be suitable as substrate for the production of the enzymes in industrial scale; however, the above study may open a new vista for sustainable management and utilization of the waste fungal biomass generated in microbial bioprocessing sectors for production of commercially viable enzymes which also endorses the principle of circular economy.

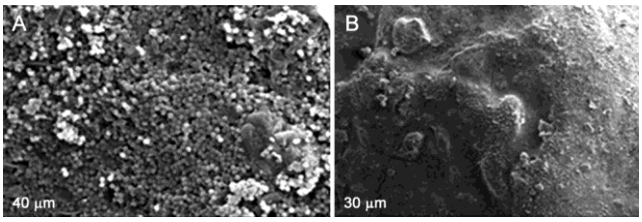

Fig. 2 — SEM analysis of fungal biomass before (A) and after (B) fermentation

Utilization of fungal biomass for for proteo-chitinolytic products formation by enzymatic hydrolysis

During microbial fermentation, different low molecular weight products were generated from large substrate molecule by the action of secreted enzymes which are subsequently utilized by the fermenting microorganisms for their growth and reproduction. Therefore little amount of the digested products are accumulated in the medium during the course of fermentation. On the contrary, to get reasonable amount of the low molecular weight hydrolyzed products, enzymatic treatment is often preferred over fermentation. As stated earlier, fungal biomass contains considerable amount of protein and chitin which upon degradation produced amino acids, soluble peptides, amino sugars (N-acetylglucosamine, glucosamine and their oligomers). These hydrolyzed products have immense applications as nutrient, antioxidant, antimicrobial, immunostimulant, plant growth promoter and so forth[41-44].

With the focus to get the aforesaid products, the dried fungal biomass of *A. niger* SKN1 was also treated with the crude enzyme cocktail preparation recovered after fermentation at optimized condition. Like fermentation, for attainment of maximum amount of desired products of interest, optimization of enzymatic hydrolysis is desirable in techno-economical perspective. In the present study, the effect of four process parameters viz. incubation time, incubation temperature, pH and (crude) enzyme volume were studied to get optimum amount of desired products through a quadratic BBD model of RSM. After experimentation (Table 4) and canonical analysis (Tables 5 and 6), it was found that the executed model is justified. ANOVA analysis revealed the significance (P <0.0001) of the proposed model for concomitant production of amino sugars, amino acids and soluble peptides by applying the same process condition. The most significant interactions for amino acids and oligopeptide and amino sugars generation were represented in Fig. 1. Significant Model F-values, insignificant lack of fits,

Table 4 — BBD design with experimental and predicted response for chitinolytic and proteolytic product production utilizing fungal biomass

Run no.	A	B	C	D	Y1		Y2	
					Actual	Predicted	Actual	Predicted
1	180	35	5	1	24	24.54	15	15.5
2	180	35	7	1.5	26	27	17	16.88
3	120	40	7	0.5	21	21.04	13	13.5
4	120	35	9	0.5	17	16.29	13	12.63
5	120	35	7	1	32	33.6	19	19
6	120	40	7	1.5	24	23.21	15	15.5
7	60	35	7	0.5	11	11	7	7.37
8	60	30	7	1	10	10.96	6	6.13
9	120	30	5	1	19	19	14	14.38
10	60	40	7	1	12	12.96	7	7.13
11	120	35	7	1	34	33.6	19	19
12	180	35	7	0.5	29	28.83	18	17.88
13	60	35	7	1.5	13	14.17	10	10.38
14	120	35	7	1	34	33.6	19	19
15	120	30	7	1.5	24	23.21	16	15.5
16	120	35	7	1	34	33.6	19	19
17	120	30	7	0.5	24	24.04	16	15.5
18	120	35	5	1.5	18	18.46	15	15.13
19	120	35	5	0.5	17	17.79	14	14.13
20	120	40	9	1	15	16	12	11.88
21	60	35	9	1	9	7.71	6	5.5
22	120	35	7	1	34	33.6	19	19
23	120	30	9	1	15	16	9	9.88
24	180	40	7	1	26	24.79	14	13.63
25	60	35	5	1	7	5.21	6	5.5
26	120	35	9	1.5	18	16.96	14	13.63
27	120	40	5	1	16	16	11	10.38
28	180	30	7	1	31	29.79	17	16.63
29	180	35	9	1	18	19.04	12	12.5

[A: Incubation time; B: Incubation temp.; C: pH; D: Enzyme vol.; Y1: Amino sugars; and Y2: Amino acids and soluble peptides]

less than 10% CV values, close proximity of predicted and adjusted R^2 values (<0.2), high AP values (>4) collectively indicated the justification and validation of the proposed model. Through numerical optimization and point prediction tool the predicted conditions for maximum enzymatic hydrolysis of fungal biomass into amino sugars, amino acids and soluble peptides were 148.41 min of incubation at 34.16°C and at pH 6.77 with enzyme volume of 0.98 mL.

Experimental validation of the model prediction was carried out by performing enzymatic catalysis at that condition and the results (35.54 and 20.06 µg/mL of amino sugars, and amino acids and soluble peptides, respectively) was in good agreement with the predicted responses. At the optimized condition the total antioxidant activity of the hydrolysate was estimated in terms of free radical scavenging activity, and about 14.12 and 17.19 µg/mL of ascorbic acid equivalent ABTS and DPPH activity were estimated, respectively. The production of different chitinolytic products was estimated through HPLC where

Table 5 — ANOVA and other statistical indices for chitinolytic product production optimization through BBD

Source	Sum of Squares	df	Mean Square	F-value
Model	1890.41	14	135.03	84.83
A	705.33	1	705.33	443.14
B	6.75	1	6.75	4.24
C	6.75	1	6.75	4.24
D	1.33	1	1.33	0.8377
AB	12.25	1	12.25	7.7
AC	16	1	16	10.05
AD	6.25	1	6.25	3.93
BC	2.25	1	2.25	1.41
BD	2.25	1	2.25	1.41
CD	0	1	0	0
A^2	446.85	1	446.85	280.75
B^2	208.9	1	208.9	131.25
C^2	810.04	1	810.04	508.92
D^2	165.42	1	165.42	103.93
Residual	22.28	14	1.59	
Lack of Fit	19.08	10	1.91	2.39
Pure Error	3.2	4	0.8	
Cor Total	1912.69	28		

Model R^2 = 0.9883, Adjusted R^2 = 0.9767, Predicted R^2 = 0.9399, Adequate Precision =31.2909, P_{Model} <0.0001, Lack of Fit = not significant, CV = 5.98%.

$Y_{Amino\ sugars}$ = 33.6+7.67A-0.75B-0.75C+0.3333D-1.75AB-2AC-.25AD+0.75BC+0.75BD+0CD-8.3A^2-5.67B^2-11.17C^2-5.05D^2

Table 6 — ANOVA and other statistical indices for proteolytic product production optimization through BBD

Source	Sum of Squares	df	Mean Square	F-value
Model	508.99	14	36.36	119.76
A	216.75	1	216.75	714
B	3	1	3	9.88
C	6.75	1	6.75	22.24
D	3	1	3	9.88
AB	4	1	4	13.18
AC	2.25	1	2.25	7.41
AD	4	1	4	13.18
BC	9	1	9	29.65
BD	1	1	1	3.29
CD	0	1	0	0
A^2	162.16	1	162.16	534.18
B^2	63.34	1	63.34	208.66
C^2	117.16	1	117.16	385.95
D^2	4.97	1	4.97	16.36
Residual	4.25	14	0.3036	
Lack of Fit	4.25	10	0.425	
Pure Error	0	4	0	
Cor Total	513.24	28		

Model R^2 = 0.9917, Adjusted R^2 = 0.9834, Predicted R^2 = 0.9523, Adequate Precision = 34.0688, P_{Model} <0.0001, $P_{Lack\ of\ Fit}$ = 0.0797, CV = 4.08%

$Y_{Amino\ acids\ and\ soluble\ peptides}$ = 19+4.25A-0.5B-0.75C+0.5D-1AB-0.75AC-1AD+1.5BC+0.5BD+0CD-5A^2-3.13B^2-4.25C^2-0.875D^2

N-acetylglucosamine and its oligomeric forms (up to degree of polymerization of 4) were predominant products (Fig. 3). Several essential amino acids like methionine, threonine, tryptophan, etc. were

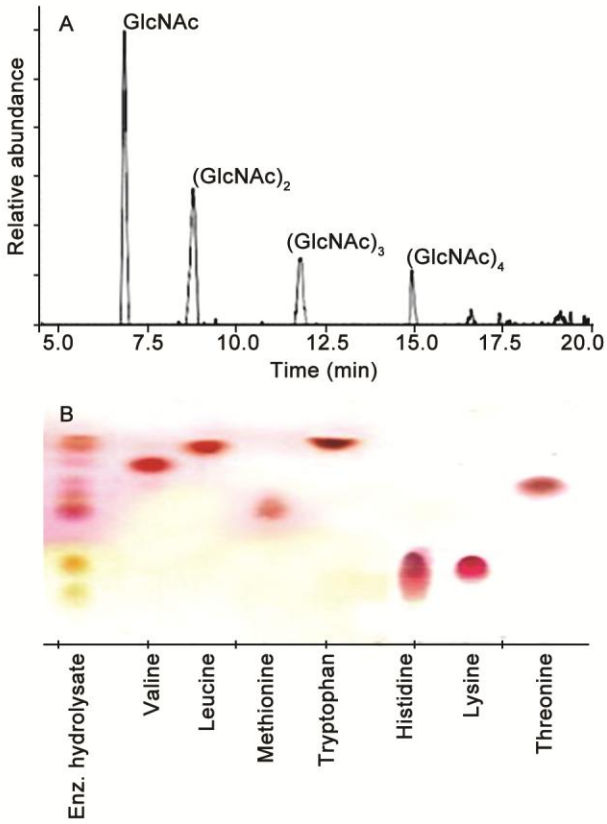

Fig. 3 — (A) HPLC analysis of chitin degraded products; and (B) TLC analysis of protein degraded products generated during enzymatic hydrolysis

accumulated in the production medium which was revealed through TLC analysis (Fig. 3). Antifungal and fungal protoplast generation potential of bacterial chitinase was previously demonstrated which principally due to degradation of chitin in fungal cell wall into N-acetylglucosamine and its oligomer[24,45-46]. A combination of bacterial and insect chitinolytic enzymes for the production of N-acetylglucosamine from mycelia of *A. niger* was reported by Zhu *et al.*[47].

The biomass of filamentous fungi is a low value sustainable alternative protein source used as feed ingredient for fish and poultry[45]. Karimi *et al.*[45] reported nutritional properties of pure filamentous fungal biomass of *A. oryzae*, *Neurospora intermedia* and *Rhzopus oryzae* as novel alternative protein sources in fish feed. The biomass derived protein (as much as 45%) from these sources are considered as high quality and contain essential amino acids like arginine, lysine, methionine and threonine. In the present study, different amino acids (including essential amino acids) and soluble peptides are generated due to enzymatic digestion of fungal

biomass which increases the nutritive value and digestibility of the biomass hydrolysate-based feed. In this context, the presence of reasonable amount of amino sugar attribute to the biomass hydrolysate as excellent antioxidant cum nutrient source.

In forest ecosystem, nitrogen-rich dead fungal biomass is considered as unique substrate that contains easily decomposable and recalcitrant compounds[33]. The oligomeric product (chitooligosaccharides, COS) of chitin hydrolysis elicits plant and arbuscular mycorrhizal (AM) interactions by symbiotic signalling pathway that regulates related gene expression[48]. Moreover, COS is reported to promote AM establishment, nodulation, PGPR growth enhancer, and therefore may act as biofertilizer[24,49-50].

It is noteworthy that for large scale production of N-acetylglucosamine and COS, shrimp shell is the first choice due to its high chitin content. However, season-based availability of shrimp shell and the environmental issues related with the conventional chitin extraction process (like aggressive acid treatment) are the two bottlenecks. Though chitin content of fungal biomass is comparatively less (10-26% as a chitin-glucan complex), such cumbersome extraction process is not necessary and the availability of fungal biomass is not an issue[49]. COS generation from lyophilized fungal biomass was reported by Crosino *et al.*[49]. Food grade COS production by utilizing waste fungal biomass of *A. niger* recovered after citric acid production was reported by Lv *et al.*[51]. An array of medical applications of COS and N-acetylglucosamine are reported till date due to their biocompatibility, water solubility, cationic, and antioxidative properties[6-7, 52] and therefore the present study opens a vista to for cost-effective production of COS from dead fungal biomass. The aforesaid studies are comparable with our approach for comprehensive utilization of post-fermented biomass of fungi.

Conclusion

Based on the acquired results, it can be concluded that the chitin and protein rich waste fungal biomass could be a robust alternative bioresource for production of microbial chitinase and protease by proteo-chitinolytic *Alcaligenes faecalis* SK10. Moreover, bioactive hydrolyzed products like amino sugars, free amino acids and soluble peptides can be extracted from the fungal biomass by the action of the enzyme cochtail. The biosynthesized enzymes and their catalytic products have paramount documented employment in diverse sections. The projected utilization strategy of the zero-valued, waste fungal biomass could offer a sustainable and eco-friendly route for production of microbial metabolites and large scale execution of the same could be proficient and endorses the principle of circular economy and waste valorization.

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Conflict of interest

Authors declare no conflict of interests.

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