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A N I N T E R N A T I O N A L J O U R N A L

Studies on Production of Single Cell Protein by *Aspergillus niger* in Solid State Fermentation of Rice Bran

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ABSTRACT

An attempt was made to apply the solid state fermentation (SSF) for the production of single cell protein (SCP) using oil free rice bran waste as substrate. A local isolate of Aspergillus niger*, was used as protein source for the studies. Total proteins were extracted to estimate the mycelial biomass from the moldy bran. Carbonate-bicarbonate extraction buffer and a pH 10 was found to be most efficient among the buffers used for the extraction of the proteins from the organism. The effect of supplementation by various sources of nitrogen and mineral solution on the final biomass yield was compared. The influence of C/N ratio on the protein yield was also studied. Sodium nitrate at C/N ratio of 1.387 was found to be an effective nitrogen-supplementing source, as it gave the higher biomass yield.*

Key words: Single cell protein, Solid State Fermentation, Aspergillus niger, Rice bran, C/N ratio

INTRODUCTION

Solid state fermentation (SSF) is growth of microorganisms on predominantly insoluble substrate where there is no free liquid. Generally, under combined conditions of low water activity and presence of intractable solid substrate, fungi show luxuriant growth. Mycelial tips of fungi have immense turgor pressure, which assist in their penetration of hard substrate. Hence, proper growth of fungi in SSF gives much higher concentration of the biomass and higher yield when compared to submerged fermentation.

The advantage in SSF process is the unique possibility of efficient utilization of waste as the substrate to produce commercially viable products. The process does not need elaborate pre-arrangements for media preparation. The process of SSF initially concentrated on enzyme production. But presently, there is worldwide interest for single cell protein (SCP) production due to the dwindling conventional food resources (Pandey & Soccol, 1998; Tengerdy, 1985; Rodriguez-Vazquez *et al.*, 1992; Zadrazil & Puniya, 1995; Nigam & Singh, 1994).

MATERIALS AND METHODS

Microorganism: A bcal isolate of *Aspergillus niger*, from decaying wood in the soil, was used for the experimental studies. The culture was

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maintained on PDA slants at 28±2°C. The subculturing was done once in a fortnight.

Substrate preparation: Oil free rice bran waste was procured from a local rice mill. It was pretreated to remove lignin and to expose the inner cellulose fibers to cellulolytic attack by the organism. The pretreatment process was carried out by adding one liter of 1% sodium hydroxide to 100 g of rice bran waste and autoclaving at 121°C and at 15 psi for 30 min. The pretreated rice bran was allowed to cool and subsequently filtered and washed to neutral pH. Then it was then dried at 60°C in an oven for 12 h. The dried bran was milled and sieved to obtain 50-mesh size and kept ready for SSF process.

Inoculum: Spores were harvested from a week old *A. niger* in five ml of sterile distilled water. Two ml of spore suspension was added to the pretreated rice bran based medium.

Fermentation medium and analytical methods:
Standardization of protein extraction buffer: Different buffers namely; citrate (pH 5.0), phosphate (pH 7.0) and carbonate-bicarbonate (pH 10.0) were used for the extraction of total proteins from *A. niger*. The organism was cultured by submerged fermentation in Modified Czapeck Dox Medium (MCD medium) in order to obtain the mycelium. The medium contained the following ingredients (g/100 ml): K_2HPO_4, 0.12; $MgSO_4$, 0.06; $FeSO_4$, 0.05; KCl, 0.02; $NaNO_3$, 0.3 and sucrose, 3.0. Four sets of 100 ml each of MCD media was inoculated by *A. niger*. These were kept on a shaker at 120-150 rpm and 28±2° C for eight days before harvesting.
The fungal mycelia were harvested by centrifugation at 2500 rpm. It was then homogenized at 6000 rpm using citrate buffer and centrifuged subsequently at 6000 rpm for 45 min. The temperature during the course of extraction was maintained at 4°C. The supernatant obtained was used for estimating protein content. Same procedure was adopted for carbonate-bicarbonate buffer and also for phosphate buffer.
Sodium hydroxide solution was also used for extraction of proteins in addition to the buffers. 0.1N sodium hydroxide was added to the weighed mycelial mass followed by boiling at 80° C for 5 min and then was centrifuged at 6000 rpm and 4°C to obtain the supernatant.

Growth of *Aspergillus niger* by solid state fermentation:
The mineral solution and different nitrogen sources were added in various combinations to 500 ml Erlenmeyer's flasks containing 10 g of pretreated rice bran (Table 1). Mineral solution was prepared by mixing $ZnSO_4.7H_2O$, 0.008 g; $FeSO_4.7H_2O$, 0.008 g and $CuSO_4.7H_2O$, 0.008 g in 100 ml of 0.2 N HCl. The nitrogen stock solutions were prepared by adding 0.05 g of the compound i.e., sodium nitrate, ammonium nitrate and ammonium sulphate and 10 ml of distilled water respectively. The flasks were made in triplicates and autoclaved at 121°C at 15 psi for 20 min. These were inoculated with 2 ml of spore suspension of *A. niger*. The contents of the flask were tapped gently to mix the spore suspension in the fermentation medium. All these flasks were incubated at 28±2°C for eight days.
The contents of each flask were harvested, weighed separately and homogenized in a homogeniser. Carbonate-bicarbonate buffer was used during the homogenization. The homogenized contents of sample from each flask were centrifuged at 6000 rpm for 45 min. The supernatant volume was measured and used for further analysis.
For estimation of total sugars, one g of the substrate was suspended in 60 ml of distilled water. This was kept at an ambient temperature for 12 h for the extraction of sugars as soaking makes the bran softer. The filtrate was then analyzed for sugars (Moniruzzaman, 1996) by using anthrone reagent (Ashwell, 1957). The biomass was expressed in terms of total protein content. Protein estimation was done by Folin method of Lowry *et al.* (1951).

RESULTS AND DISCUSSIONS

Rice bran is one of the most abundant and locally available agricultural wastes. Rice bran showed higher carbon content after pretreatment with alkali (carbon content = 3.458 mg/g of rice bran).
Among the extraction buffers used carbonate-bicarbonate buffer at pH 10 was found to be most efficient in extracting out total proteins of *A. niger*. The protein yield with various buffers was as follows (mg/g of sample): citrate buffer, 3.403; phosphate buffer, 6.351; carbonate-bicarbonate,

6.884 and sodium hydroxide (0.1N), 3.154. The biomass in terms of the final protein content of the moldy bran harvested on the eighth day is shown in the Table 2 and Fig.1.

Rice bran when supplemented with mineral solution and nitrogen sources individually or together improved the biomass yield (expressed in terms of total protein yield). Among all these combinations, the supplementation of rice bran with sodium nitrate gave the highest protein yield. Supplementation of rice bran with mineral solution individually also improved the biomass yield. But the biomass yield was not as high as that with the sodium nitrate supplementation. Individual nitrogen sources in combination with mineral solution supported the biomass yield to a lesser extent as compared to sodium nitrate or mineral solution (Table 2).

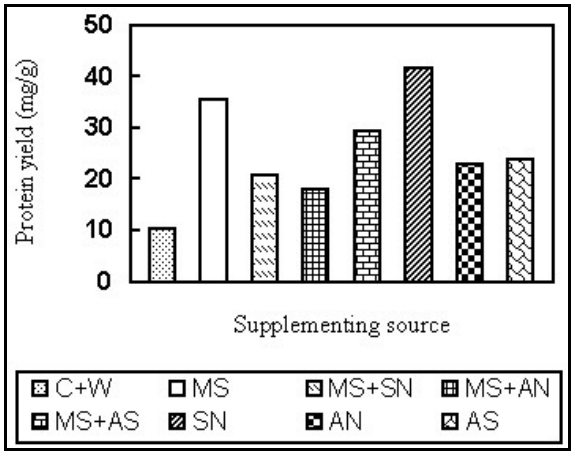

Figure 1 - Effect of supplementation on the final protein yield

Where, C+W (Control + Water), MS (Mineral solution), MS+SN (Mineral solution + Sodium nitrate), MS+AN (Mineral solution + Ammonium nitrate), MS+AS (Mineral solution + Ammonium sulfate), SN (Sodium nitrate), AN (Ammonium nitrate) and AS (Ammonium sulfate) are different media compositions provided for the cultivation of the biomass

The relation between C/N ratio and final biomass yield (in terms of protein yield) is given in Table 2. The change in C/N ratio was observed due to supplementation. The C/N for pretreated rice bran was 1.729. The highest biomass yield was obtained when the C/N was 1.387. The high biomass yield was also observed for C/N 1.729 when mineral solution supplemented the medium. But the results in Table 3 indicated that C/N ratio was not the only factor controlling the final biomass yield. This was because when same C/N ratios were maintained by various sources the protein yield was not similar. Even under the conditions of same C/N, sodium nitrate still favours the organism to grow better giving a higher biomass yield when compared to other nitrogen sources.

From the above observations it is clear that the availability of the nitrogen is the major controlling factor in the final biomass yield. Supplementation with mineral solution individually, gives higher yield because it provides continuous acidic medium to support the growth. As the organism grows in this condition it produces more of cellulases. These enzymes make available more of glucose monomers from the rice bran in the medium as well as help in the release of nitrogen for the growth.

The biomass yield was the highest when sodium nitrate supplements the medium alone. But when it was used along with the mineral solution its free availability for the growth seems to be hindered. Therefore, the protein yield, which was around 41.68 mg/g of rice bran, drops to almost half to 22.88 mg/g of rice bran. The contributing factor for the drastic reduction in the biomass yield could be traced to the reactivity of sodium ion. Sodium is placed higher than all the metals in mineral solution viz., zinc, ferrous, cuprous and hydrogen ions in the activity series of metals. Hence, it displaces all these metals from their salts. The nitrate ion released from sodium nitrate in the medium (due to the above principle of displacement) reacted with the free cations of the mineral solution and formed nitrates. As a result, free nitrogen and also mineral solution controlling the pH was not available as before to support the growth of *A. niger*.

Ammonium nitrate or ammonium sulphate when supplemented the medium along with mineral solution gave higher protein yield when compared to medium supplemented by sodium nitrate along with mineral solution. This was because the activity of mineral solution was not hindered, as ammonium ion formed was weak and hence unable to replace the metal ions in mineral solution. So the mineral solution controlled the pH and helped in the release of glucose as explained above and ammonium ion supplemented the nitrogen simultaneously.

Table 1 - Composition of the fermentation media used

Flask number	Pretreated rice bran (g)	Mineral solution	Nitrogen source (from the stock solution)
Control	10	Not added	Not added
Control with water	10	Not added	Not added
Sample 1	10	6 ml	Not added
Sample 2	10	5 ml	1 ml $NaNO_3$
Sample 3	10	5 ml	1 ml NH_4NO_3
Sample 4	10	5 ml	1 ml $(NH_4)_2SO_4$
Sample 5	10	Not added	6 ml $NaNO_3$
Sample 6	10	Not added	6 ml NH_4NO_3
Sample 7	10	Not added	6 ml $(NH_4)_2SO_4$

Table 2 - Final protein yield in various media combinations

Contents of the flask	Protein yield (mg/g of moldy rice bran)	Net protein yield (mg/g of rice bran)	C/N ratio
Control	2.00	0	1.729
Control with water	12.48	10.48	1.729
PRB*+MS**	37.44	35.44	1.729
PRB+MS+ $NaNO_3$	22.88	20.88	1.66
PRB+MS+ NH_4NO_3	20.08	18.08	1.589
PRB+MS+$(NH_4)_2SO_4$	31.20	29.20	1.639
PRB+ $NaNO_3$	43.68	41.68	1.387
PRB+ NH_4NO_3	24.96	22.96	1.134
PRB+$(NH_4)_2SO_4$	26.00	24.00	1.300

* Pretreated rice bran ** Mineral Solution

Table 3 - Final protein yield at same C/N ratio but differ nitrogen source

Contents of the flask	Net protein yield (mg/g of rice bran)	C/N ratio
Control (PRB+water)	0	1.729
PRB*+MS**	34.2	1.729
PRB+MS+ $NaNO_3$	22.4	1.387
PRB+MS+ NH_4NO_3	20.93	1.387
PRB+MS+$(NH_4)_2SO_4$	30.03	1.387
PRB+ $NaNO_3$	40.91	1.387
PRB+ NH_4NO_3	22.09	1.387
PRB+$(NH_4)_2SO_4$	24.10	1.387

** Pretreated rice bran ** Mineral Solution*

List of revisions/modifications made in the manuscript as suggested by the reviewer:

Query	**Title, Paragraph, Line etc.**	**Response**
Anupama - Is the name complete	Title-Author names	Yes
What size? How?	Heading: Materials and Methods Subheading: Substrate preparation Penultimate line	The dried bran was milled and sieved to obtain 50 mesh size. (Change incorporated in the text)
For what?	Heading: Materials and Methods Subheading: Fermentation medium & analytical methods 2nd paragraph, 10th line	The necessary corrections were incorporated in the text.
Conditions of centrifugations?	Heading: Materials and Methods Subheading: Fermentation medium & analytical methods 3rd paragraph, 7th line	----for 5 min and then was centrifuged at 6000 rpm & 4°C to obtain the supernatant------ Incorporated in the text
Why?	Heading: Materials and Methods Subheading: Growth of *Aspergillus niger* by solid state fermentation Penultimate paragraph, 4th line	This was kept at an ambient temperature for 12 h for the extraction of sugars as soaking makes the bran softer.
Tables 1,2,3 & 5	General comments	Deleted as per the instructions and the summarized matter incorporated in the text at appropriate places.
Was the strain of *A. niger* isolated by the authors or obtained from some source?	General comments	The strain was isolated locally from the decayed wood lying in the soil. Mentioned in 'Materials and Methods' ---'Micro-organism' section.
Is there any accession number for it?	General comments	No (Not available with us as the organism being locally isolated)
Changes in the Title of paper	Title	p-changed to 'upper case' from-replaced by 'by' by-replaced by 'in'
Addresses of Institutions not as per the journal guidelines	Addresses of the authors	Changed as per directions and author guidelines
Email address of the corresponding author	Addresses of the authors	Deleted form the author address title and incorporated as 'footnote' on the same page
Submerged fermentation----------alternative techniques.	Abstract, 1st paragraph, 1st line	Deleted as required
Is	Abstract, 1st paragraph, 4th line	Replaced with 'was'
Technique	Abstract, 1st paragraph, 4th line	Replaced with 'solid state fermentation'
Single Cell Protein	Abstract, 1st paragraph, 4th line	S, C & P were made lower case as directed
Aspergillus niger	Abstract, 1st paragraph, 5th line	Made 'not italic'
which grows well on lignocellulosic wastes	Abstract, 1st paragraph, 5th -6th line	Deleted as suggested
The effect----	Abstract, 2nd paragraph , 1st line	Shifted and made continuous with the 1st paragraph as directed
Italicized '*Keywords--'*	Keywords	Made 'normal & not italic'

(Cont.)

List of revisions/modifications (cont.).

Numbering in front of all the heading viz., Introduction, Materials and Methods etc., etc.	All the Headings and subheadings	Removed the numbers as well as the tab space as directed. Also the paragraphs started with a 'tab space' as directed in the manuscript.
Solid State Fermentation	Heading: Introduction 1st paragraph, 1st line	S & F made 'lower case' as directed
Hence, proper------to submerged fermentation	Heading: Introduction 1st paragraph, last line	Full stop incorporated
The advantage-----	Heading: Introduction 2nd paragraph, 1st line	Tab space at the start of new paragraph.
Single Cell Protein	Heading: Introduction 2nd paragraph, 9th line	S, C & P made lower case as directed
Page numbers	All pages	Deleted as instructed
Spacing between the text columns and other text margins, spacing etc.	All pages	Made as per the journal guidelines and instructions in the original (revised) manuscript.
Selected organism	Heading: Materials and Methods Subheading: Selected organism	Changed to 'micro-organism:' as directed
A local isolate-----	Heading: Materials and Methods Subheading: Selected organism	Shifted as directed in the text
Cultures were	Heading: Materials and Methods Subheading: Selected organism 3rd line	Changed to 'culture was'
The cultures----sporulation'	Heading: Materials and Methods Subheading: Selected organism Last line	Deleted as directed
Substrate preparation	Heading: Materials and Methods Subheading: Substrate preparation	Changed to 'Substrate preparation:'
Oil free----	Heading: Materials and Methods Subheading: Substrate preparation	Shifted as directed
12hours	Heading: Materials and Methods Subheading: Substrate preparation 12th line	Changed to '12 h' as required
Inoculum	Heading: Materials and Methods Subheading: Inoculum	Changed to 'Inoculum:"
A week old-----	Heading: Materials and Methods Subheading: Inoculum Start of the paragraph	Changed to 'Spores were harvested from a week old *A. niger* in five ml of distilled water. Two ml of spore suspension was added---------.' As directed in the original text. The starting sentence was also shifted as required
Standardization of protein extraction buffer:	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer:	The sub subheading was made italic as required. 1st sentence of the paragraph shifted as required

(Cont.)

List of revisions/modifications (cont.).

Aspergillus niger	Throughout the manuscript	Changed to *A. niger* at all places as directed
For this purpose	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 1st paragraph, 3rd line	Deleted as required
4 sets	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 1st paragraph, 7th line	Changed to 'Four'
From 7day old PDA slants	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 1st paragraph, 9th line	Deleted as directed
The fungus was obtained-----growth and was	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 2nd paragraph, 1st line	Modified to 'The fungal mycelia were harvested-----
2500rpm	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 2nd paragraph, 4th line	Changed to '2500 rpm'
The pellet was weighed	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 2nd paragraph, 4th line	Deleted as needed
The homogenized sample was	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 2nd paragraph, 6th line	Replaced with 'and'

(Cont.)

List of revisions/modifications (cont.).

As this solution -------- conventionally	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 3rd paragraph, 3rd line	Deleted as required
5min	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Standardization of protein extraction buffer: 3rd paragraph, 7th line	Changed to '5 min'
The carbon----rice bran.	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Growth of *Aspergillus niger* by solid state fermentation 2nd paragraph, 1st line	Deleted as required
Tables 4, 6 & 7	Tables	Changed as per the journal guidelines i.e., The lines deleted as instructed, Upper case letters made to lower case in the Table title row, Table was adjusted as per single spacing and serial numbers were deleted as directed.
To remove the debris	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Growth of *Aspergillus niger* by solid state fermentation 3rd paragraph, 7th line	Deleted as required
1gm	Heading: Materials and Methods Subheading: Fermentation medium and analytical methods Sub subheading: Growth of *Aspergillus niger* by solid state fermentation 4th paragraph, 1st line	Changed to 'one g'
mg/gm in Fig. 1	Heading: Results and discussions Y-axis	Changed to 'mg/g'
It is used as sole carbon source in SSF studies	Heading: Results and discussions 1st paragraph, 2nd line	Deleted as required
Has contained	Heading: Results and discussions 1st paragraph, 4th line	Replaced with 'showed'
Rice bran is---------- provided to the medium.	Heading: Results and discussions 4th paragraph, 1st line	Deleted as directed
Nitrogen source	Heading: Results and discussions 5th paragraph, 2nd line	Deleted as directed

(Cont.)

List of revisions/modifications (cont.).

,	Heading: Results and discussions 5th paragraph, 5th line	Deleted as directed
Is	Heading: Results and discussions All the paragraphs Heading: Conclusions All the paragraphs	Changed to 'was' where directed and needed.
Indicates	Heading: Results and discussions 8th paragraph, 1st line	Changed to 'indicated'
However, the type of nitrogen source used----------controlled the final yield. The substrate, rice bran------	Heading: Conclusions 1st paragraph	Changed to 'However rice bran when supplemented ------'
Rice Bran, is one of the most----------near future.	Heading: Conclusions Last paragraph	Deleted as directed
References	Heading: References	Changed as per the journal guidelines and as per the instructions in the text

CONCLUSIONS

A higher yield of SCP production from *A. niger* was possible by SSF of rice bran. Though, utilization of SSF for SCP production is emerging field, encouraging results are obtained and some success is achieved in improving the overall protein yield by supplementation of rice bran based SSF medium. The C/N ratio was not very effective in controlling biomass yield but the supplementation of rice bran with various nitrogen sources or mineral solution separately or in combination improved the *A. niger* growth. The biomass yield was best with rice bran based medium with sodium nitrate as the nitrogen source for single cell protein production.

ACKNOWLEDGEMENT

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RESUMO

Uma tentativa foi feita para aplicar fermentação no estado sólido (SSF) para a produção de “single cell protein”(SCP utilizando o resíduo livre de óleo farelo de arroz, como substrato. Uma cepa de *Aspergillus niger* isolada do local foi usada como fonte de proteína para os estudos. As proteínas totais foram extraídas para estimar a biomassa micelial do farelo fermentado. A condição mais eficiente encontrada para extração das proteínas do microrganismo foi feita com tampão Carbonato-bicarbonato pH 10. Comparou-se o efeito da suplementação do substrato com várias fontes de nitrogênio e de solução no rendimento final da biomassa. Também estudou-se a influência da relação de C/N no rendimento da proteína. A suplementação mais eficaz encontrada foi com nitrato de sódio com uma relação C/N de 1,387, resultando em maior rendimento de biomassa.

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