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# Studies on *Aspergillus oryzae* Mutants for the Production of Single Cell Proteins from Deoiled Rice Bran

*Rudravaram Ravinder*[1], *Linga Venkateshwar Rao*[1] *and Pogaku Ravindra*[2]*

[1]Department of Microbiology, Osmania University, Hyderabad 500 007, India
[2]University College of Technology, Osmania University, Hyderabad 500 007, India

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**Summary**

Ethyl methyl sulphonate was used to induce point mutation in *Aspergillus oryzae* (MTCC 1846). Incubation with ethyl methyl sulphonate for 1 h resulted in 98 % killing of spores. By screening the survived colonies three hypermorphs were found (Shan1, Shan2 and Shan3). These three mutants along with the *A. oryzae* (MTCC 1846) were used for the production of single cell proteins. They grew profusely on deoiled rice bran and produced higher percentage of protein. Among the three mutants Shan2 had higher protein content in the pH range of 3–7 and temperatures 36–45 °C, maintaining low quantities of nucleic acids. The specific growth rate of Shan2 was higher on the media containing substrates like glucose, maltose and cellulose. Shan2 had higher content of amino acids when compared with FAO standard and *A. oryzae* (MTCC 1846), but the amino acid content of Shan2 was approximately equal to that of soyabean meal. The comparative protein content of Shan2 and *A. oryzae* (MTCC 1846) was 57 and 43 %, respectively, while their nucleic acid content was 3 and 7.2 %, respectively. Protein enrichment of 18.9 % in Shan2 resulted from the cultivation on deoiled rice bran as compared to protein content of the *A. oryzae* (MTCC1846) indicating that Shan2 possessed good amenability for SCP production.

*Key words*: solid state fermentation, mutants screening, deoiled rice bran, single cell protein, *Aspergillus oryzae* (MTCC 1846), Shan1, Shan2 and Shan3 mutants

## Introduction

India is an agrarian country where 2/3 of its population depend on rice as staple food. On average 4 million tonnes of rice bran, 0.6 million tonnes of rice bran oil and 0.2 million tonnes of deoiled rice bran (DOB) waste are generated annually containing 39 % of cellulose and 9 % of protein (SEA 1996). Therefore, it is felt that there is an urgent need to utilize DOB to convert cellulose into protein for food and feed by solid state fermentation technology.

A fermentation process on solid support, which has low moisture content (*1*), characterizes Solid State Fermentation (SSF). It is a low-level technology, which uses a reduced reactor volume per unit of converted substrate, where fungi were applied to obtain desirable product (*2*). Fungi have been used as a source of protein in specialty food for centuries and *Aspergillus* sp. has been widely used for SCP production (*3*). SSF has been exploited for production of feed (*4*) and food (*5*). The ever-increasing protein demands and recent high prices of soyabean meal have further necessitated the search for an alternative means of cheap and economic agroresidue for protein enrichment (*6*).

* Corresponding author; E-mail: dr_ravindra@hotmail.com

In this study, deoiled rice bran, a locally available industrial waste, was used as a substrate for the production of SCP. Mutation studies were carried out on *A. oryzae* (MTCC 1846) for enhancement of protein production.

## Materials and Methods

### *Microorganisms*

*A. oryzae* (MTCC 1846) was obtained from the strain collection of IMTECH, Chandigarh, India. Mutant strains of *A. oryzae* were termed as Shan1, Shan2 and Shan3.

Chemicals and raw material used were: ethyl methyl sulphonate (EMS) (Sigma) and deoiled rice bran (DOB) from a local rice mill.

### *Media preparation*

DOB was treated with 0.25M NaOH and autoclaved at 121 °C for 15 min. The pretreated DOB was allowed to cool and subsequently filtered with muslin cloth and washed under running water to neutral pH. Then it was dried at 60 °C in an oven, kept overnight and then milled to fine particles of 0.3 mm (50 mesh size).

### *Inoculum preparation*

*A. oryzae* (MTCC 1846) and its mutants were maintained on potato dextrose agar (PDA) subcultured once a month and stored at 4 °C. Spores were harvested from a-week-old PDA slants with distilled water.

### *Mutation of A. oryzae (MTCC 1846) with EMS*

About $10^9$ spores per mL of *A. oryzae* (MTCC 1846) were collected from one-week-old slant and 300 μL ethyl methyl sulphonate (EMS) was added. This was incubated at equal time intervals. The spores were diluted, spread on PDA and incubated for 60 h at 30 °C. The survival percentage was calculated from the number of colonies obtained at each time interval. Spores incubated for an hour were used for screening as suggested by Hopwood *et al.* (*7*).

### *Fermentation*

An amount of 10 g of pretreated DOB was taken in 500-mL flask and 80 % moisture was maintained by adding inoculum medium containing per 1 L of distilled water: $FeSO_4 \cdot 7H_2O$ 0.1 g, $MgSO_4 \cdot 7H_2O$ 5 g, $(NH_4)_2SO_4$ 3 g and $K_2HPO_4$ 1 g. This was autoclaved at 121 °C for 15 min. An amount of 3 g of each glucose, cellulose and maltose were added to 100 mL of inoculum medium in separate flasks and pH was adjusted to 6. In one set of flasks *A. oryzae* (MTCC 1846) was added and in another Shan2 mutant was added. Each flask was inoculated with spores $3 \cdot 10^9$/mL of Shan2 mutant and *A. oryzae* (MTCC 1846) and incubated in stationary conditions for 3 days at 42 and 30 °C, respectively. The effect of temperature was studied by incubating DOB at different temperatures at constant pH=4 for Shan2 and pH=7 for *A. oryzae* (MTCC 1846). Each sample was removed for biomass separation by filtration at regular intervals of 10 h. The contents of the flask were transferred into carbonate bicarbonate buffer as described by Anupama *et al.* (*8*). Further, the biomass of mutant Shan2 and *A. oryzae* (MTCC 1846) grown on pure cellulose was used for determination of amino acid composition.

### *Optimization of pH*

The pH of the inoculum used to moisten DOB was adjusted to be in the range of 2–9. About 8 flasks were inoculated with Shan2 mutant and *A. oryzae* (MTCC 1846) with an amount of spores $3 \cdot 10^9$/g of DOB and incubated at 42 and 30 °C, respectively. All the experiments were carried out in triplicates.

### *Analytical methods*

Protein was measured by method of Lowry (*9*), total nitrogen was determined by microKjeldahl method (*10*), while lignin and cellulose by the gravimetric method of Van Soest (*11*). Nucleic acids were determined after heat-shock treatment of biomass at 52.5 °C for 2 h by the method suggested by Norris and Ribbon (*12*) and the composition of amino acids was analyzed with 6M HCl at 110 °C with Biocal Automatic amino acid analyzer.

## Results and Discussion

The kinetics of mutagenesis of *A. oryzae* (MTCC 1846) was carried out at different time intervals and the survival percentage of *A. oryzae* (MTCC 1846) shows 2 % survival of spores during 1-hour incubation (Fig. 1), after which rapid killing of spores was observed and at 70 min of incubation no spores survived. By screening the survived spores three hypermorphs, Shan1, Shan2 and Shan3 were detected on the basis of their protein content. Mutants (genetic variants) were found to give higher yield of product. In this study Shan2 was used as the source for SCP production since it contains higher protein content (57 %) and low nucleic acid content (3 %) compared to *A. oryzae* as indicated in Table 1 (*13*).

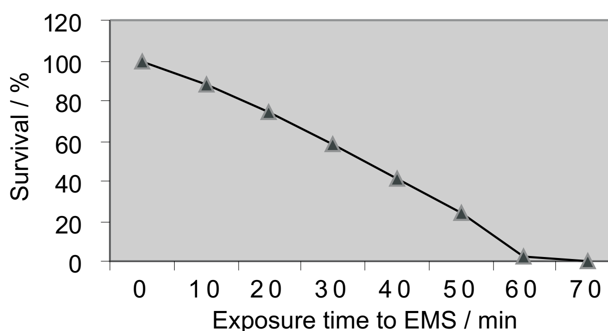


**Fig. 1. Survival curve of *A. oryzae* (MTCC 1846) treated with EMS**

Table 1. Comparison of total protein content and nucleic acids (expressed as mass fraction) in the biomass of mutant strains Shan1, Shan2, Shan3 and *A. oryzae* (MTCC 1846)

| Composition | *w*/% | | | |
|---|---|---|---|---|
| | Shan1 | Shan2 | Shan3 | *A. oryzae* (MTCC 1846) |
| Protein (N · 6.25) | 52 | 57 | 55 | 43 |
| Nucleic Acids | 3.6 | 3 | 3.8 | 7.2 |
| DNA | 0.7 | 0.5 | 0.8 | 1.5 |
| RNA | 2.9 | 2.5 | 3 | 5.8 |

The comparative growth rate of Shan2 mutant and *A. orzyzae* (MTCC 1846) showed that Shan2 had maximum specific growth rate (μmax) when grown on glucose, maltose and cellulose. The growth rate of Shan2 mutant and *A. oryzae* (MTCC 1846) was higher on media with glucose than that on maltose and cellulose (Table 2). The growth rate was reduced in maltose and cellulose due to the need for maltose and cellulose to be hydrolyzed into monomers. Since Shan2 mutant showed higher growth rate on pure cellulose, it was applied on DOB for its protein enrichment.

Table 2. Comparison of maximum specific growth rates ($\mu_{max}$/h) of *A. oryzae* (MTCC 1846) and Shan2 mutant cultivated on media containing different substrates

| Substrate | Specific growth rate/($\mu_{max}$/h) | |
|---|---|---|
| | Shan2 | *A. oryzae* (MTCC 1846) |
| | $\mu_{max}$/h | $\mu_{max}$/h |
| Glucose | 0.231 | 0.203 |
| Maltose | 0.198 | 0.173 |
| Cellulose | 0.165 | 0.119 |

Fig. 2 shows that higher yield of protein was observed at the optimum temperature in the range of 28–30 °C for *A. oryzae* (MTCC 1846) and 38–45 °C for Shan2 mutant. The wider range of optimum temperature of Shan2 mutant would be beneficial for large-scale production of single cell proteins and it may be ideal for subtropical places like India. The major limitation of poor heat dissipation in SSF can be overcome by Shan2 mutant (*14*). Moreover, this improved characteristic of higher protein yield at high temperatures could be more advantageous for industrial scale fermentation.

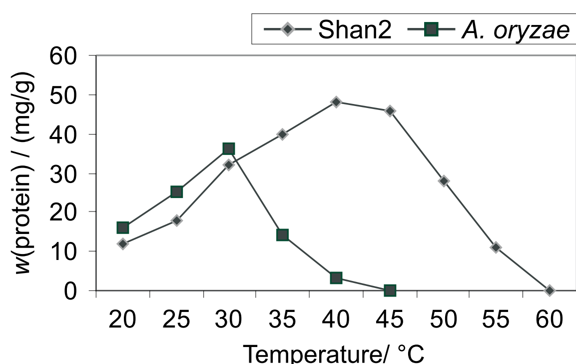


**Fig. 2. Effect of temperature on the protein content of *A. oryzae* (MTCC 1846) and Shan2 mutant grown on DOB at the 30$^{th}$ h of incubation**

Fig. 3 shows that the optimal growth of *A. oryzae* (MTCC 1846) was in the pH range of 5–7, while Shan2 mutant has an optimum pH range between 3–7. Shan2 can grow at relatively low pH (optimum at 4), which is desirable for the production of pure culture at industrial level where there is a high risk of contamination.

From Fig. 4 it was observed that the content of amino acids of Shan2 was higher than that of the wild type and FAO standard, except for the content of cysteine. Amino acid content of Shan2 mutant is comparable with soyabean meal. This is why we can prefer cheaper source like DOB for SCP production.

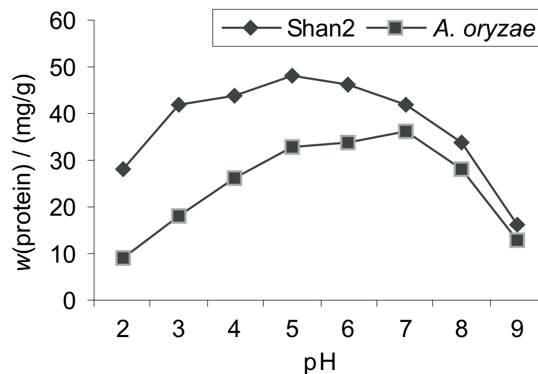


**Fig. 3. Effect of pH on the protein content of *A. oryzae* (MTCC 1846) and Shan2 mutant grown on DOB at the 30$^{th}$ h of incubation**

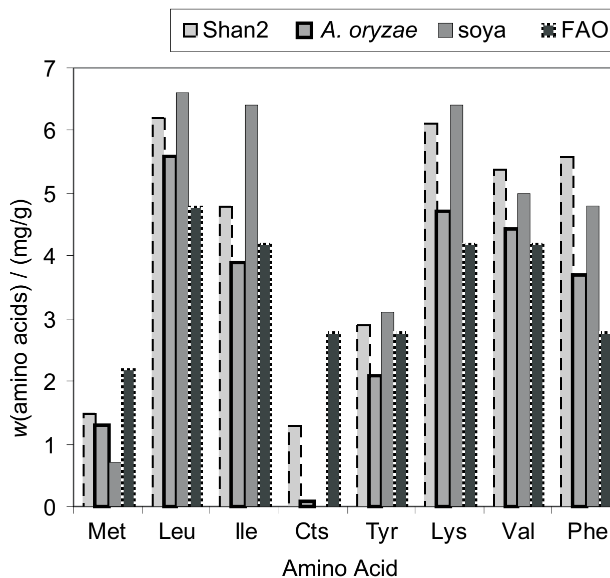


**Fig. 4. Comparison of amino acid composition of Shan2 mutant with FAO standard, soyabean meal and *A. oryzae* (MTCC 1846) grown on cellulose**

Table 3. Comparison of protein content and essential amino acids of pretreated DOB (% dry matter) and of *A. oryzae* (MTCC 1846) and Shan2 grown on DOB (60 h of incubation)

| Components | Fraction of dry matter/% | | |
|---|---|---|---|
| | Pretreated DOB | *A. oryzae* on DOB | Shan2 on DOB |
| Total nitrogen | 9.2 | 16.4 | 28.1 |
| Cellulose | 39 | 24 | 18 |
| Hemicellulose | 31 | 24.2 | 23.8 |
| Lignin | 4 | 4.7 | 5.2 |
| Tryptophan | 0.14 | 0.38 | 0.86 |
| Lysine | 0.46 | 2.3 | 4.1 |
| Threonine | 0.12 | 0.92 | 1.64 |
| Cysteine | 0.24 | 0.41 | 0.72 |

Comparing the protein content and essential amino acids of DOB (% dry matter) with *A. oryzae* (MTCC 1846) and Shan2 grown on DOB (Table 3), it was observed that total nitrogen content of Shan2 mutant was increased from 9.2 to 28.1 %, which is much greater than of *A. oryzae* (MTCC 1846) (from 9.2 to 16.4 %) as well as the increase of the content of lysine, threonine, cysteine and tryptophan.

## Conclusion

DOB was used as potential source for products with higher protein content by utilizing cellulose present in DOB. An attempt was made to mutate *A. oryzae* to increase the conversion of cellulose into protein. Three of its hypermorphs (Shan1, Shan2 and Shan3) were identified and Shan2 was found to give better yield of protein with lower content of nucleic acids. Overall results indicate that Shan2 mutant can be used as potential strain for SCP production. Since DOB was successfully utilized for the enrichment of protein in product, there is a possibility of converting agroresidue waste to proteinaceous feed and food. Moreover, the results confirm that the enrichment of DOB with inorganic nitrogen source is to enhance the protein content of the product by converting inorganic into organic nitrogen.

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# Istraživanje mutanata *Aspergillus oryzae* za proizvodnju proteina u jednostaničnim organizmima uzgojenim na rižinim mekinjama iz kojih je uklonjeno ulje

**Sažetak**

Etilmetil-sulfonat inducirao je točkastu mutaciju u *A. oryzae*. Inkubacijom od 1 sata u prisutnosti etilmetil-sulfonata uništeno je 98 % spora. Selekcijom preživjelih kolonija pronađena su tri hipermorfa (Shan1, Shan2 and Shan3), koja su zajedno s *A. oryzae* (MTCC 1846) korištena za proizvodnju proteina u jednostaničnim organizmima. Ti su mutanti snažno rasli na rižinim mekinjama iz kojih je uklonjeno ulje. Od tri mutanata, Shan2 je sadržavao veći udjel proteina u pH-području od 3 do 7 i pri temperaturi od 36 do 45 °C, uz manju količinu nukleinskih kiselina. Specifična brzina rasta Shan2 bila je veća u podlogama koje su sadržavale glukozu, maltozu ili celulozu. Udjel aminokiselina u Shan2 bio je približno isti kao u sojinom brašnu, iako viši u usporedbi s FAO standardom i *A. oryzae* (MTCC 1846), dok je količina proteina u Shan2 i *A. oryzae* iznosila 57 odnosno 43 %, a nukleinskih kiselina 3 odnosno 7,2 %. Uzgojem na rižinim mekinjama iz kojih je uklonjeno ulje, Shan2 je proizveo 18,9 % više proteina nego *A. oryzae*, što pokazuje da je Shan2 važan za proizvodnju proteina u jednostaničnim organizmima.