Effect of Extracellular Factors on Growth and Dimorphism of *Rhizopus oryzae* with Multiple Enzyme Synthesizing Ability

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Abstract *Rhizopus oryzae* PR7 MTCC 9642 was a dimorphic fungus that showed a regular 90 days cycle of filament (mycelium) to pellet (yeast) transformation through a distinct bottom dwelling intermediate state and the pellets never revert back to filamentous form. Apart from the normal cycle, high temperature (37°C and above) and extreme pH also induced the yeast formation. Among the ions tested, calcium and chloride ions were found to restore the filamentous morphology, even in extreme pH and temperature. Cysteine HCl also played noteworthy role in maintaining mycelial growth even at adverse condition. Immobilized spores showed the appearance of intermediate form instead of typical yeast form even at high temperature. The strain could produce a number of extracellular hydrolytic enzymes like cellulolytic, xylanolytic, pectinolytic and amylolytic enzymes. The pellet and mycelial forms were found to be a better producer of cellulase–lignocellulase enzymes and amylolytic enzymes respectively, which might be correlated with their infectivity. Increase in inoculum size, agitation during cultivation, change in carbon and nitrogen source failed to induce mycelial growth in extreme conditions, which might be explained as irreversible change of configuration of protein responsible for mycelial development.

Keywords Dimorphism · Pseudohypha · Filaments · Pellet form · *Rhizopus oryzae*

Introduction

Many filamentous fungi present the phenomenon of dimorphism [1], where a monokaryotic yeast form alternates with a dikaryotic hyphal form [2]. Interest in this phenomenon derives from the prevalence of dimorphism among the pathogenic fungi [3]. The strategy of dimorphism is extensively exploited by both plant and animal pathogenic fungi, where the encounter with the host prompts a shift in the mode of growth [4]. This is an active approach to survive in a changing environment, which simply shift their thallic organization to adapt and thrive in the new conditions. Dimorphism is studied in many fungi, *Histoplasma capsulatum* [5], *Blastomyces dermatitidis*, and *Sporothrix schenckii* [6], *Ceratocystis ulmi* [7], *Paracoccidioides brasiliensis* [8], *Candida albicans* [9], *Penicillium marneffei* [10], *Rhizopus oryzae* ATCC 20344 [11]. Mucor dimorphism has interested microbiologists since the time of Pasteur [12]. Among these Mucorales fungi, *R. oryzae*, although is the most common causative agent of zygomycosis, is widely used for the production of fermented foods, industrial enzymes, organic acids, corticosteroids and alcoholic beverages [13].

Fungal pellet formation is an important topic of fermentation research as filamentous growth increases the viscosity of the medium, requiring a higher power input to maintain adequate mixing. They may wrap around impellers, foul agitation blades, and block spargers [14]. Therefore from industrial point of view pellet formation is warranted for commercial production of fermented products. It has been reported that many factors such as agitation, medium nutrients, pH, polymer additives, and inoculum size influence the formation of fungal pellets. However, a few studies on the effects of all of these factors on fungal pellet formation have been reported [15].

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This paper conducted a comprehensive investigation on various factors effecting dimorphism in a filamentous fungus, *R. oryzae* PR7 with a normal filament-pellet cycle of 90 days and a potential producer of several extra cellular cellulolytic, amylolytic and other enzymes.

Materials and Methods

Micro Organism

Rhizopus oryzae PR7 MTCC 9642, a producer of extra cellular endoglucanase [16] and isoamylase [17] was isolated from the decaying vegetation enriched soil of West Bengal, India. The strain was identified by and deposited to Microbial Type Culture Collection, India.

Chemicals

All chemicals used were of analytical grade.

Cultivation of the strain

The strain was cultivated in 100 ml Erlenmeyer flasks each containing 20 ml Basal Medium (BM) composed of (gl^{-1}): peptone 0.9; $(\text{NH}_4)_2\text{HPO}_4$ 0.4; KCl 0.1; $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ 0.1; (pH: 6). Starch 0.5 or carboxymethyl cellulose (CMC) 0.5 was used as the sole carbon source.

Preparation of Inoculum

The fungus was grown in 1% PDA plate for 48 h at 28–30°C. The inocula were prepared by making hyphal discs (0.5 cm diameter). Each disc was used to inoculate 20 ml of medium [18].

Measurement of Growth

Growth of the mycelial type culture was measured by weighing the dried (at 80°C for 120 min) mycelial mat on dried pre weighed filter paper (Whatman No 1) growth of the yeast form was measured by turbidometric method at 650 nm in a spectrophotometer [19].

Effect of Various Factors

The culture flasks (100 ml) with 20 ml medium was cultivated statically and in shaking conditions at various temperatures (4–40°C) for 48 h. To check the effects of temperature, pH, inoculum size, additives (10 mM) and antifungal agents (0.25 $\mu\text{g/ml}$) the factor to be investigated was changed or added keeping all other factors constant.

Enzyme Assay

The culture broth was filtered through filter paper (Whatman No 1) followed by centrifugation at 10,000 rpm for 5 min and the supernatant was used as the crude enzyme. The endoglucanase activity [16] and amylase [17] activities were measured by dinitrosalicylic acid method [20].

To check the β -glucosidase, polygalacturonase, salicinase, xylanase activities, 1% (w/v) cellobiose, pectin, salicin and oat spelt xylan was used as respective substrate in the assay mixture.

Immobilization Method

The fungal spore suspension (1 ml) prepared from each hyphal disc of 0.5 cm diameter was immobilized through ionotropic gelation in 5% (w/v) strontium alginate gel beads [21]. The beads containing entrapped cells were stabilized for 60 min, washed thoroughly and kept at 0°C. Both the free spores and immobilized spores were grown in BM at 30–40°C.

Photomicrographic Study

The cotton blue-lactophenol stained and the acridine orange stained mycelia [22] were visualized under Axioscop-40 (Zeiss) microscope at 100 $\times$. For SEM, paraformaldehyde–glutaraldehyde fixed and totally dehydrated specimens were sputter coated with gold palladium under vacuum and observed and photographed in a scanning electron microscope (FEI Quanta-200 MK 2).

All the experiments were done in triplicate and their values were averaged.

Results and Discussion

The strain showed two distinct morphological variations—a filamentous mycelial form and yeast like ‘pellet’ form (Fig. 1). A chain like pseudohypha made up of yeast like cells was found to remain present encircling the mycelium (Fig. 1A). After a normal period of 90 ± 10 days or after addition of additives, change in temperature and pH, the mycelial form turned into yeast like state (Fig. 1B) through an intermediate form. The intermediate forms showed a preference to be deposited at the bottom of the culture broth indicating their affinity towards anaerobic conditions, but the fully formed yeast was found to remain uniformly distributed throughout the broth. The yeast form, once formed was never returned to the mycelial form; even the subcultures of yeast would give rise to yeast form only.

Fig. 1 Scanning electron micrograph of **A** mycelial form and **B** yeast form of *R. oryzae* PR 7

Effect of Time

After a normal approximately 3 months cycle the filamentous forms turned into yeast like form. The filaments with fully formed spores were gradually broken down releasing star shaped mucilaginous droplets. In course of time, although initially few sporangiospores could be seen, later the entire culture was occupied by yeast cells (Fig. 2A, B).

Effect of Temperature

The strain after 48 h incubation, showed filamentous growth (Fig. 3) at lower temperature (4–34°C), but yeast like growth at higher temperature range (40–48°C). A filamentous culture grown at 28°C for 24 h, if transferred to

higher temperature or kept at 4°C, an intermediate form along with yeast type was found to form at the bottom of the culture broth, keeping the surface filaments apparently unchanged. The dimorphic transition from mycelium to yeast (M-Y) was triggered by a temperature shift from 25 to 37°C and was critical for pathogenicity [23, 24]. In the present strain, room temperature (28–30°C) provided a condition for mycelial development, while increased temperature (37°C and above) brought about a prompt shift from mycelial–yeast form, probably due to some altered programme of gene expression. It did not revert back to the mould or filamentous form even incubated at lower temperature, a result totally opposite to the observation from *Arxula adeninivorans* [25]. This was assumed to be as a result of some irreversible nature of protein change.

Fig. 2 **A, B** Gradual transformation of spore to yeast type cell through a star shaped fluid filled intermediate phase

Fig. 3 Effect of temperature on the dimorphism and relative growth of *R. oryzae* PR7

Effect of Immobilization

In contrast to the normal yeast like growth at higher temperature, immobilized spores showed few intermediate forms. The tendency of immobilized spores to develop an intermediate form instead of a well defined yeast form at 37°C indicated that immobilization increased the thermostability [26] of those proteins related to the dimorphic changes of the strain.

Effect of pH

The present strain showed filamentous growth at a pH range of 4–8, but at extreme pH 2 and 9 and at higher temperature, yeast type of growth was found (Fig. 4.). At higher temperature, only yeast type of growth was found which indicated that temperature was the more dominant factor than pH for yeast formation, an observation not

Fig. 4 Effect of pH on dimorphism and relative growth of *R. oryzae* PR7

similar to that of *Yarrowia lipolytica* [27]. Although, generally a lower initial pH value in the cultivation medium was inhibitory to fungal growth [28], in the present strain, at 28°C, change in pH led to the gradual change from filamentous to yeast form (Fig. 5).

Effect of Inoculum

It was found that the size of inoculum had a positive correlation with the mycelial mat formation but could not protect the mycelia to yeast transformation at higher temperature. The sporangiospores from young to middle aged fungal culture gave rise to filamentous growth, a report which went in agreement with that from *Mucor lusitanicus* [29]. Inoculation with yeast type of inocula resulted in only yeast type of culture. On the other hand, failure of induction of a mycelial type of growth at higher temperature, even in presence of a higher inoculum size ruled out the existence of any type of “quorum sensing molecules” (QSMs) [2].

Effect of Agitation

Although anaerobiosis activate formation of yeast like cells [30], aeration by cultivation at a rotary shaker (160 rev/min) led to vigorous yeast like growth both at 28 and 37°C.

Effect of Carbon Source

Starch, glycogen, and cellulose could induce remarkable growth than other carbon sources. The increase in concentration of carbon sources (0.5–1% w/v) could not resist the appearance of yeast form at higher temperature (Table 1) a report contrary to that of *Mucor circinelloides* var *lusitanicus* [31].

Effect of Nitrogen Source and Amino Acids

Peptone and yeast extract acted as best growth supplementing nitrogen sources and among the amino acids tested, only cysteine HCl could restore the filamentous form even at higher temperature (Table 2), which might be due to restoration of mitochondrial respiration that would otherwise stop during transformation to yeast phase [5, 32]. Sub culturing the strain in cysteine HCl supplemented media successfully prevented the appearance of yeast form both at higher temperature and during the normal transition of mycelia–yeast form. This finding was comparable to the observation in *Ceratocystis ulmi*, where proline induced the yeast morphology whereas ammonium, arginine, or asparagine induced the mycelial state [33].

Fig. 5 A–D Gradual transformation of filamentous form to yeast type pellets in *R. oryzae* PR 7

Table 1 Effect of various carbon sources on relative growth, dimorphism and enzyme production by *R. oryzae* PR7

Carbon sources (0.5–1% w/v)	Preferred temperature (°C)	Morphology	Relative growth (%)	Produced enzyme	Maximum production at (h)
CMC	37	Intermediate-pellet	65	Endoglucanase	48
Starch	28	Filament (spore)	100	Amylase	72
Glycogen	28	Filament (spore)	100	Amylase	72
Amylopectin	28	Filament	90	Amylase	72
Amylose	28	Filament	75	Amylase	72
Dextrin	28	Filament (spore)	87	Amylase	72
Pectin	37	Pellet	60	Polygalacturonase	80
Xylan	37	Intermediate	45	Endoxylanase	96
Cellobiose	37	Pellet	70	β -glucosidase	48
Salicin	37	Pellet	56	Salicinase	48
Lactose	28–37	No growth	ND	ND	–
Glucose	28–37	Filament (spore)	80	ND	–

Filament (spore): filament with large number of spores, Filament: filament with little spore, *ND* non detectable. 100% yeast and intermediate type: 0.98 at A_{650} ; 100% filament: 0.184 g

Table 2 Effect of nitrogen sources and additives on morphology and relative growth of *R. oryzae*

Additives		Relative growth at 30 ± 2°C (%)		Relative growth at 40 ± 2°C (%)
Peptone	F	100	Y	100
Tryptone	F	80	Y	80
Yeast extract	F	100	Y	100
Urea	F	20	Y	20
Beef extract	F	30	Y	25
NaCl	F	100	F	80
CaCl ₂	F	100	F	100
SrCl ₂	F	95	F	78
Ca acetate	F	100	F	100
Ca lactate	F	97	F	80
Malic acid	I	40	Y	40
Succinic acid	F	35	I	45
Ascorbic acid	F	32	I	20
Cyclohexamide	ND	–	ND	–
Griseofulvin	F	15	I	20
Fluconazole	F	19	I	16
Cysteine HCl	F	100	F	98
Histidine HCl	F	95	Y	86
Tyrosine	F	78	Y	34
Phenyl alanine	F	70	Y	36

100% yeast and intermediate type: 0.98 at A₆₅₀; 100% filament: 0.184 g

F filament, I intermediate, Y pellet type, ND no growth detected

Effect of Additives

Out of several salts tested, only CaCl₂, SrCl₂ and to a lesser extent NH₄Cl restored the filamentous growth at 37°C. This might be due to the restoration of proper cell volume

and maintenance of resting membrane potential by activated chloride channels, which was generally activated by Ca²⁺ [34], a report similar to the filament formation of *Zymomonas mobilis* [35] but contrary to that from *R. oryzae* [3], *Aspergillus niger* [1], *P. marneffei* [9]. Addition of Griseofulvin (mitotic inhibitor) and Flucanazole (cytochrome P450 1,4- α -demethylase inhibitor) caused appearance of intermediate and filamentous form respectively at 28°C but yeast like form at 37°C when the pseudohyphae got detached from the mycelium releasing a large number of yeast like cells (Fig. 6).

Effect of Morpho Types on Enzyme Production

It was found that the intermediate-pellet form was better producer of lignocellulolytic enzymes whereas mycelial form was suitable for amylase synthesis (Table 2). Highest level of endoglucanase and amylase production was achieved within 48 and 72 h of growth respectively. The solid state culture encouraged the restoration of filamentous growth and spore formation, whereas in submerged culture the strain became more susceptible to environmental changes (data not shown).

Concluding Remark

Although various adverse conditions like higher temperature, extreme pH, additives force *R. oryzae* to undergo a transformation from filamentous to yeast like pellet form, a regular cycle of this transformation was found to exist even in presence of favourable conditions. In presence of cysteine HCl and/or CaCl₂ this transformation could be stopped. The strain probably tends to produce an infective form that waits for a suitable host (*Oryza sativa*) which grew

Fig. 6 Gradual detachment of pseudohypha, release of mucilaginous material and pellet formation (observed under fluorescent microscope)

faster with a higher competence in extracellular cellulase/ligno cellulase production in the first or second phase and eventually showed amylase activity to consume the starchy part of the attacked tissue.

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