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Effect of Agitation and Aeration Rates on Chitinase Production Using *Trichoderma virens* UKM1 in 2-l Stirred Tank Reactor

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Abstract Shrimps have been a popular raw material for the burgeoning marine and food industry contributing to increasing marine waste. Shrimp waste, which is rich in organic compounds is an abundant source of chitin, a natural polymer of *N*-acetyl-D-glucosamine (GluNac), a reducing sugar. For this respect, chitinase-producing fungi have been extensively studied as biocontrol agents. Locally isolated *Trichoderma virens* UKM1 was used in this study. The effect of agitation and aeration rates using colloidal chitin as control substrate in a 2-l stirred tank reactor gave the best agitation and aeration rates at 200 rpm and 0.33 vvm with 4.1 U/l per hour and 5.97 U/l per hour of maximum volumetric chitinase activity obtained, respectively. Microscopic observations showed shear sensitivity at higher agitation rate of the above system. The oxygen uptake rate during the highest chitinase productivity obtained using sun-dried ground shrimp waste of 1.74 mg of dissolved oxygen per gram of fungal biomass per hour at the k_La of 8.34 per hour.

Keywords Agitation · Aeration · Chitinase · *Trichoderma virens* UKM1 · Shrimp waste · Colloidal chitin

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Introduction

Shrimp waste constitutes 45–60% of the whole shrimp in the form of the head and body carapace [1], and only 25% is recovered as meat [2], whereas 69.5% of the organic weight of shrimp cuticle on average is chitin [3]. Recent major work has been done extensively using shrimp waste as the main substrate in chemical and biological processes to obtain products of higher value [4–9].

Chitin is the main constituent of shrimp wastes with β-(1–4)-linked *N*-acetyl-D-glucosamine (GluNac) as the chief building block. Having highly hydrophobic property, chitin is fundamentally inert to natural degradation and is insoluble in aqueous solutions. Owing to its abundant and cheap resource and biocompatibility, chitin has the potential for bioconversion to simpler molecules of GluNac monomers and chito-oligosaccharides by means of enzyme-catalyzed reactions or chemical procedures with the ease in production coming from the former procedure [3].

Chitinases, belonging to the family of glycosyl hydrolases, are the enzymes responsible for the biological conversion of chitin. These enzymes find major applications in the field of agriculture [10], medicine [11], biotechnology [12], waste management [13], and industry [14].

Trichoderma sp. possesses a chitinolytic system formed by several endochitinases, several exochitinases, and several *N*-acetyl-glucosaminidases, so that the measured specific chitinase activity is the result of the hydrolytic activity of those chitinolytic enzymes that are functional under specific condition. Up till date, a number of significant studies have been performed on chitinolytic enzymes from *Trichoderma* spp., especially on *T. harzianum* in which some seven individual chitinases have been elucidated [15–17]. Felse and Panda [18] attempted *T. harzianum* as their fungus of choice and with chitin flakes as the chitinase inducer in a defined salt medium for chitinase production in a 1-l stirrer tank bioreactor.

The aim of this study is to determine the effects of agitation and aeration of a 2-l stirred tank reactor (STR) on the chitinase production from *T. virens* UKM1 using colloidal chitin and sun-dried ground shrimp (SDG) waste as substrates. Colloidal chitin was designed as control substrate for comparison purposes.

Materials and Methods

Microorganism and Inoculum Preparation

The locally isolated fungus, *T. virens* UKM1, was supplied by a research collaborator from Universiti Kebangsaan Malaysia (UKM). Long-term stock culture was prepared by resuspending 0.7 ml of the stock spore solution with 0.3 ml of 80% sterile glycerol aseptically. The vials were kept at −20 °C. The fungus was grown and maintained on potato dextrose agar (PDA) plates at 30 °C, and matured spores were harvested after 7 days of incubation with sterile distilled water.

Colloidal Chitin Preparation

Chitin flakes from shrimp shell were used in the experiments. Ten grams of commercial shrimp chitin flakes were added to 100 ml of 85% phosphoric acid, stirred with a glass rod, and kept at 4 °C for 24 h. After that, 2 l of tap water was added, and the gelatinous white

material formed was washed with tap water until the filtrate had a pH of 6.5 to 7.0. The retentate is the colloidal chitin, which was drained yielding a soft, pasty consistency with 98% moisture. The colloidal chitin was sterilized at 121 °C, 15 psi for 15 min, and stored till further use.

Shrimp Waste Pretreatment

SDG were prepared from raw shrimp waste after the waste was washed, drained, and dried under the sun for 2–3 days or till dried by grinding with Waring blender, then it was sieved through a no. 80 mesh sieve at a 180-μm particle cutoff point. The sieved particles were placed under UV light overnight before use.

Seed Culture

Optimized Kawachi medium [19] was used in this study. The Kawachi medium was optimized by other researchers in the laboratory (unpublished data) for highest chitinase production as was listed in Table 1. The seed culture for cultivation into 2 l STR was prepared by pre-germinating a suspension of spores in 150 ml optimized Kawachi medium at 30 °C for 16–18 h to give the total inoculum concentration in 1.5 l of culture broth as 1×10^6 spores/ml.

Fermentation in 2-l Stirred Tank Reactor

The 2-l STR configuration were as follows: operating volume, 1.5 l; liquid height (H_L), 11.5 cm, and internal vessel diameter (D_t), 13 cm; ratio of H_L/D_t, 0.88; impeller diameter (D_i), 5 cm; ratio of D_i/D_t, 0.38; baffle width (B), 1 cm; ratio of B/D_t, 0.077; impeller height (W_i), 1 cm; ratio of W_i/D_t, 0.077; number of Rushton turbine impellers, 2; impeller spacing (S), 5 cm; S/D_t, 0.38; impeller height (H_i), 1 cm. Four baffles with a rounded bottom glass vessel. Fermentation temperature was adjusted at 30 °C, pH 6.0 with 120 rpm agitation speed, and aeration rate of 1.5 l/min or 1.0 vvm with a ring sparger. The pH of media was adjusted to pH 6.0 initially and throughout the experiment using 1 M H_2SO_4 and 1 M NaOH. The range of agitation speed employed was between the range of 120 and 600 rpm. The range of aeration rate was from 0.67 to 2.0 vvm.

Optimized Kawachi medium was modified (designated as M5; Table 1) without bacto-peptone and yeast extract, and was employed as the production medium in separate

Table 1 Composition of standard and optimized M4 and M5.

Composition (%)	Medium 4 (M4)		Medium 5 (M5)	
	Standard	Optimized	Standard	Optimized
Chitin	0.3	0.2	0.3	0.2
Peptone	0.5	0.35	–	–
Yeast extract	0.3	0.15	–	–
$NaNO_3$	0.2	0.16	0.2	0.16
K_2HPO_4	0.1	0.1	0.1	0.1
KCl	0.05	0.05	0.05	0.05
$MgSO_4.7H_2O$	0.05	0.05	0.05	0.05
$FeSO_4.7H_2O$	0.001	0.001	0.001	0.001

experiments with two different chitin sources (colloidal chitin and SDG waste) at a control initial pH of 6.0. Fermentation was carried out at a temperature of 27–30 °C for 8 days. All experiments were done in duplicates.

Dynamic Method of Oxygen Uptake Rate and k_La Determination

The method adapted from Shuler and Kargi [20] and Badino et al. [21] for determination of k_La in a non-fermentative system using steady-state or static gassing-out technique was done by lowering the oxygen concentration in the solution.

Sample Assays

The soluble protein concentration was determined according to a modified Lowry method [22]. A calibration curve was plotted with bovine serum albumin as the protein standard at concentrations ranging from 0 to 1.0 g/l.

The dinitrosalicylic acid (DNS) chitinase assay method is an adaptation of the chitinase assay established by Rojas-Avelizapa et al. [23].

The Remazol brilliant blue R (CCRBB) method for precise chitinase assay was adapted from Gomez-Ramirez et al. [24].

To determine the relationship of the fungal biomass and the glucose production, cell dry weight with the residual substrate was determined, as the substrate used in the fermentation process is insoluble, and it also constitutes the fungi cell wall; thus, it was taken into account because the total cell dry weight assuming the weight of the substrate did not depreciate throughout the fermentation or because the depreciation in weight was negligible. This was done by subtracting the cell dry weight and residual chitin with a control with the same amount of substrate without fungal cells.

Results and Discussion

Effect of Agitation

Agitation plays a vital role in mass transfer in a submerged fermentation. The higher the agitation, the higher the shear rate. For cells that are shear sensitive, optimization of agitation rates are important to determine its viability and growth pattern throughout the fermentation; it is also necessary to consider the formation of metabolites during the fermentation. For such metabolites with catalytic properties, enzymes, for instance, are shear sensitive and, at certain agitation rates, may render it dysfunctional due to conformational changes [25– 26].

Agitation intensity may affect both fungal morphology [27–30] and mass transfer in the bioreactor [21, 31]. In many fungal fermentation, a high agitation rate is necessary to provide adequate mixing and mass transfer, especially when the fungal cells grow in a freely dispersed form, which results in a non-Newtonian broth and a high apparent viscosity. However, mechanical forces can cause mycelia damages. Thus, the agitation rate is limited to a range that avoids exerting high shear stresses on fungal mycelia.

From Fig. 1, the highest volumetric chitinase productivity of 4.1 U/l per hour was observed at an agitation rate of 200 rpm. The productivity decreased at 240 rpm and continued at higher agitation rates. Almost no productivity was recorded at 600 rpm as confirmed by Fig. 2b that showed that the cells mostly lysed into fragmented mycelia, and

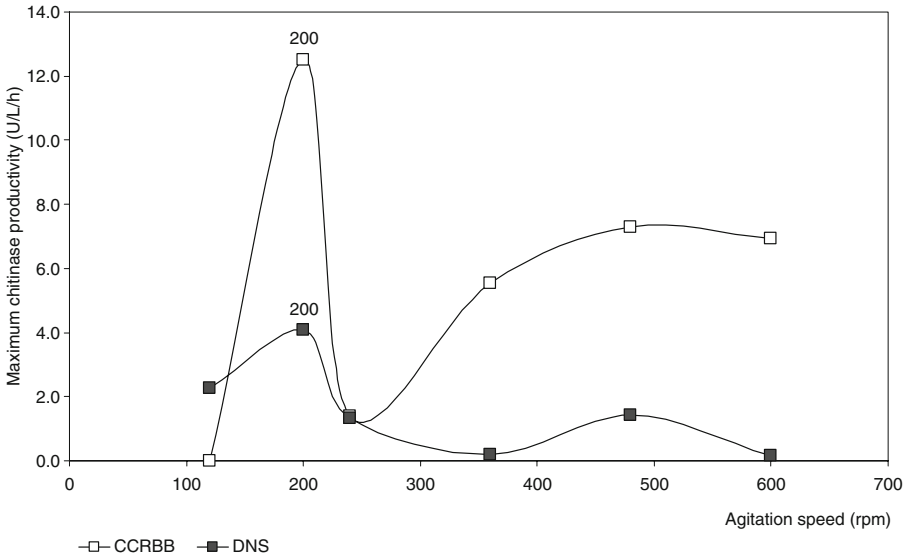

Fig. 1 Effect of agitation rates using DNS and CCRBB chitinase enzyme assay methods

no whole mycelium were observed. The reverse was recorded for the agitation speed of 200 rpm as shown in Fig. 2a, where the mycelia growth indicates fungal growth and enzyme production. Similar studies done by Felse and Panda [18] on *T. harzianum* showed that agitation rate at 224 rpm was the most suitable for cell growth and for chitinase production, which is agreeable with the results obtained above. Study on chitinase enzymes from *Trichoderma* spp. reported that they were generally extracellular enzymes excreted outside the cells [16–17, 32–33].

The relationship between the pellet size, agitation rates, and chitinase productivity were studied in this system. It was interesting to note that, over the ranges of the agitation employed, the pellet sizes showed significant differences. Papagianni [34] showed that formation of pellet in filamentous fungi was induced during submerged fermentation. Figure 3 showed the variation of pellet sizes at different agitation rates. It was observed at 200 rpm that the pellet are less compact and smaller than in 240 and 360 rpm, whereas the

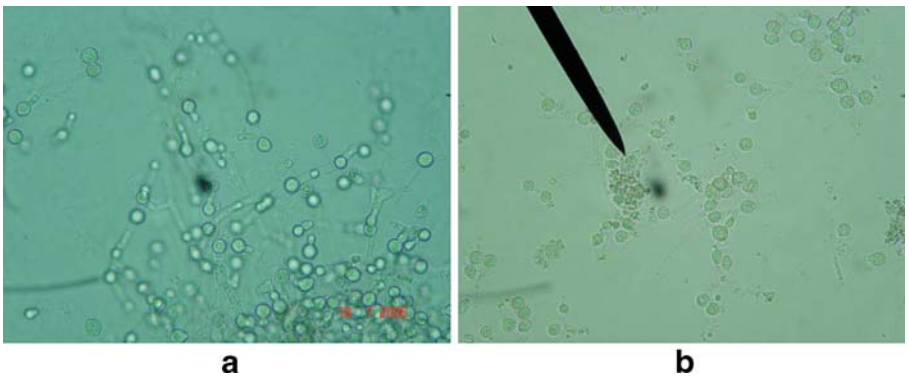

Fig. 2 Light micrograph of *T. virens* UKM1 in submerged fermentation at 400 X magnification **a** 200 rpm and **b** 600 rpm

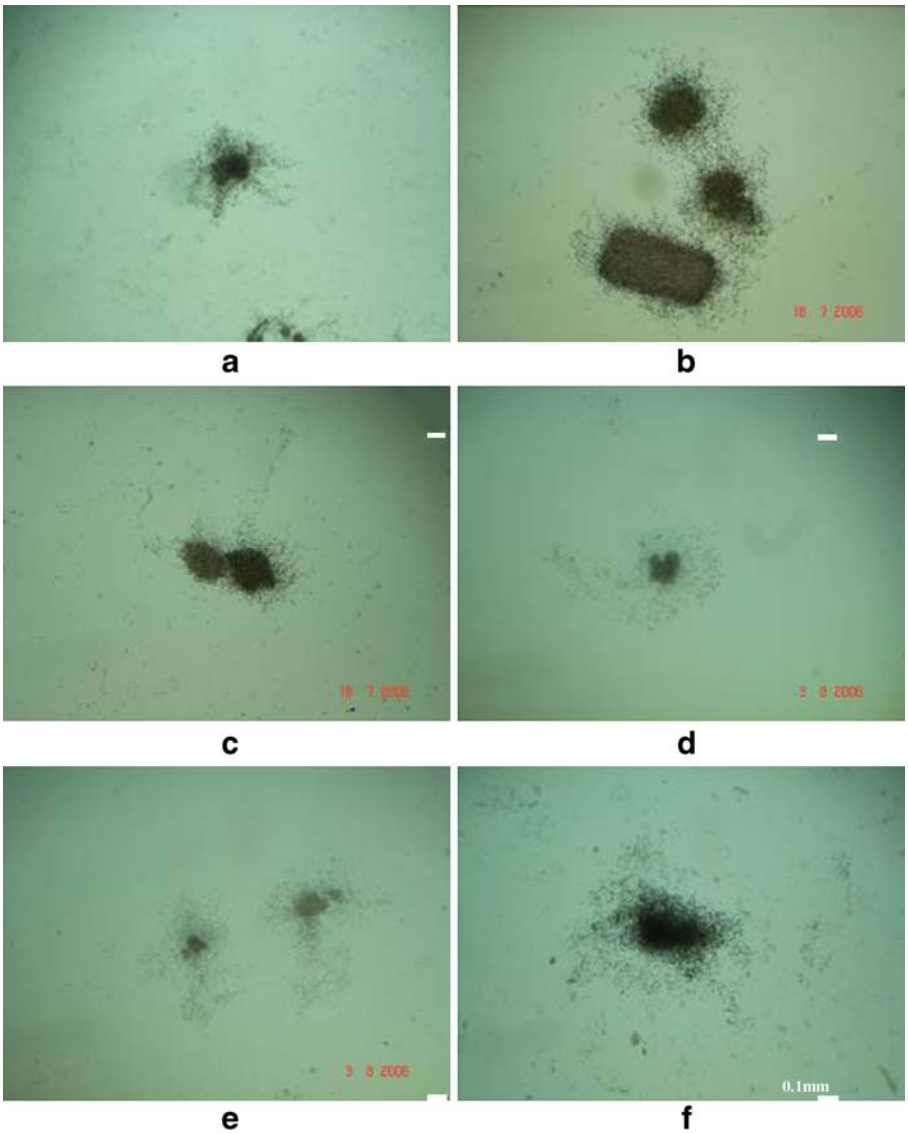

Fig. 3 Light micrograph of *T. virens* UKM1 in submerged fermentation at ×40 magnification, showing the comparison of pellet size at different agitation rates. **a** 200 rpm, **b** 240 rpm, **c** 360 rpm, **d** 480 rpm, **e** 600 rpm, **f** best conditions of 200 rpm and 0.33 vvm

pellet in 480 and 600 rpm was smaller still and less dense as compared to the pellet in 240 and 360 rpm. According to Papagianni [34], there are two formations of pellets; coagulative and non-coagulative forms. Coagulative pellets are characterized when the pellets clump together while germinating due to intertwining of hyphae. The non-coagulative pellets are a result of one spore to one pellet. In this case, the pellet formation was the coagulative form, as a high total spore concentration of 1×10^6 spores/ml was used in the seed culture for pre-germination before inoculation into the bioreactor. This was agreeable. It concurred with

our observations as the highest spore concentration employed gave high chitinase enzyme productivity (unpublished results).

The difference in pellet sizes over the range of agitation rates can be explained by the shear effect on the pellet itself. This was elaborated by Cui et al. [29] accordingly that, at high agitation rates, which resulted in high shear, hairs of the pellet were shaved off by mechanical forces, which were reseeded into mycelial growth. It was observed that both pellet and loose mycelia grew concurrently in the fermentation broth. The mechanical forces from the impellers damaged and deactivated the loose mycelia. In addition, aging and vacuolation resulted in the reduction of activity of both pellet and loose mycelia. It explained the reduction of enzyme activity at higher agitation rates. It was suggested that, as the agitation rate increase, the pellet size decrease and the lower the fraction of pellet mass in the total biomass obtained.

Effect of Aeration

Oxygen is often a limiting component in fermentations because of its low solubility and the low volumetric mass transfer in bioreactors. Some researchers used oxygen instead of air to maintain the dissolved oxygen (DO) at a higher level. The DO tension may affect productivity, cell autolysis, fungal morphology, etc. [29–30]. The effect of DO on fungal fermentation can be either positive or negative. In the culture of aerobic microorganisms, oxygen serves as a substrate for energy generation. It may enhance the cell growth and protein production directly or through changes in morphology.

The effect of DO on fungal morphology has been reported in the literature. Cui et al. [35–36] found that pellets were denser when DO was close to the saturation level. While in the case of very low DO levels, pellets were rather weak and fluffy. The biomass per wet pellet volume and the porosity of the pellets were all functions of DO and the pellet size. Smaller pellets formed under higher DO levels had a higher intrinsic strength. Wongwicharn et al. [37] reported that two distinct morphologies were observed when varying the oxygen enrichment in the gas supply. Under oxygen limiting conditions, long, sparsely branched hyphae with a low percentage of dactiveT length were formed. While under higher oxygen enrichment levels (e.g., 30–50%), shorter hyphae with more branching and a higher percentage of dactiveT length were formed. The production of both the native and foreign enzymes was correlated well with the dactiveT length or tip numbers.

Generally, most submerged fermentation employ 1 vvm as the standard aeration rate. In this study, it was found that chitinase production was best at 0.33 vvm with 5.97 U/l per hour of chitinase productivity (Fig. 4). It was also observed, as the aeration was increased, that the chitinase productivity gradually decreased. This could be attributed to the turbulence created by larger and faster air bubbles that disrupted the growing fungal mycelia and pellets. As shown in Fig. 5, there was a significant difference of the density of fungal cells at 0.33 and 2 vvm, at which in the latter, mycelia grew less dense and more dispersed. However, at different agitation rates, the pellet sizes did not show much difference at various aeration rates as shown in Fig. 6. The only difference noted was the pellet size at 0.03 vvm, which was smaller than the other pellet sizes at higher aeration rates.

Formation of Chlamydospore

It is interesting to note that the medium used in this fermentation induced chlamydospore formation of *T. virens* UKM1 as shown in Figs. 2 and 5. A chlamydospore is a thick-walled

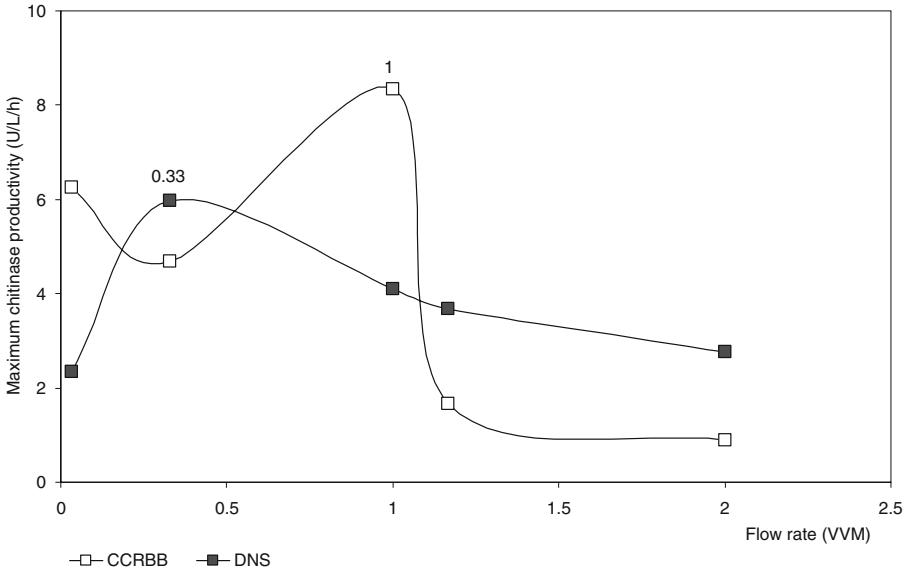

Fig. 4 Effect of aeration rates using DNS and CCRBB chitinase enzyme assay methods

mitotic spore sometimes produced secondarily from an existing cell, generally acting as a 'resting spore,' which is the dormancy state before germination to survive in unfavorable conditions. Chlamydospores are produced by members of both ascomycota and basidiomycota [38]. It was reported by Zhuang et al. [39] that chlamydospores were easily produced by *Trichoderma* sp. at low pH and DO. This, in particular, agrees with this study as the fermentation pH was maintained at pH 6.0. As observed, the chlamydospores were formed either between the hyphae or formed terminally in the hyphae tip. Similar results were obtained by Li et al. [40] while studying the biological control properties of both *Trichoderma* spp. and *Gliocladium* spp. These chlamydospores could germinate after they were moved to PDA. Essentially, they play an important role as survival structures of *Trichoderma* spp. and *Gliocladium* spp. in natural ecosystems yet little is known about the

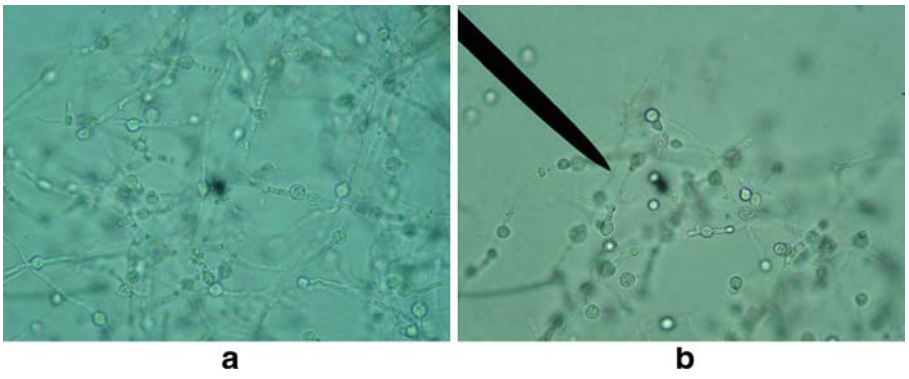

Fig. 5 Light micrograph of *T. virens* UKM1 in submerged fermentation at ×400 magnification. **a** 0.33 vvm and **b** 2.00 vvm

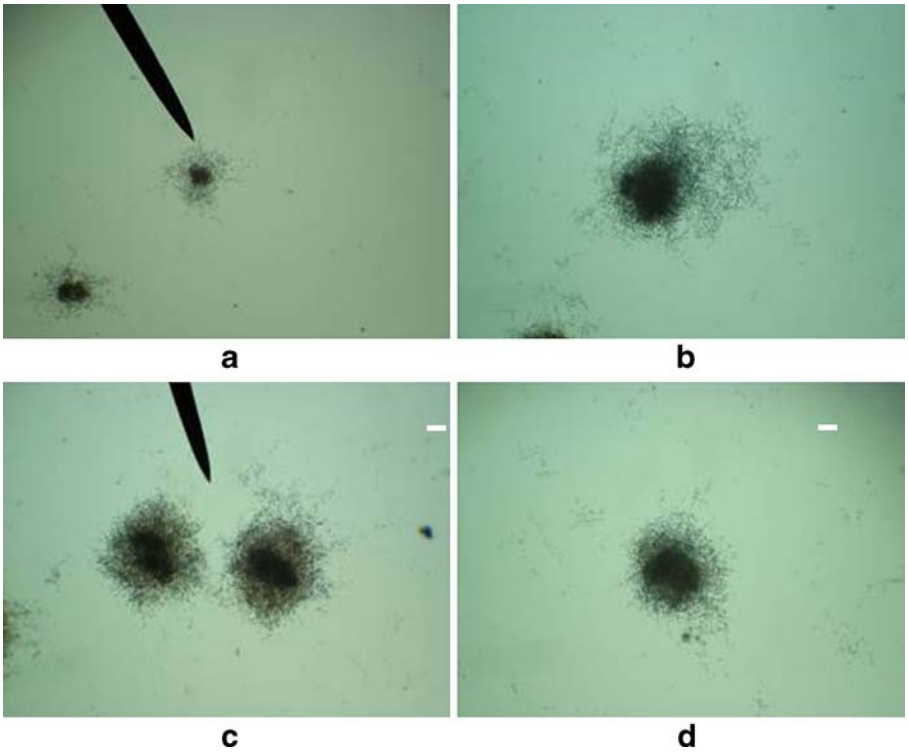

Fig. 6 Light micrograph of *T. virens* UKM1 in submerged fermentation at ×40 magnification, showing the comparison of pellet size at different aeration rates: **a** 0.03 vvm, **b** 0.33 vvm, **c** 1.17 vvm, **d** 2.00 vvm

factors that enhance their formation. This procedure could be used as an alternative mode of *T. virens* chlamydospore formation at a larger scale for biological control purposes.

k_La at Different Agitation Rate, Aeration Rate and Medium Composition

Oxygen constitutes 20.8% of air, and its solubility in liquid is much lesser. Due to its imminent importance to microbial metabolism, mass transfer studies in fermentation focuses on the mass transfer between gas and liquid phases. Therefore, the transfer is

Table 2 Volumetric mass transfer coefficient in different media, agitation, and aeration rates in 2 l STR.

Media	Agitation (rpm)	Aeration (vvm)	k_La (h^{-1})
dH_2O	200	0.33	25.63
M5SDG	200	0.33	8.34
M5CC	200	1.0	16.61
		2.0	22.54
	300	1.0	23.44
		2.0	37.95
	400	1.0	32.63
		2.0	50.84
	600	1.0	71.49
		2.0	79.37

measured by the volumetric mass transfer coefficient, k_La. The measurement is essential to establish the system's aeration efficiency and to quantify agitation rates, which are the effects of operating variables on the provision of oxygen in the liquid medium [41]. Agitation can improve the k_La. As when air enters fermentor, the rotating impellers disperse the air stream into fine bubbles, thus increasing the gas–liquid interfacial area. Agitation increases air retention time and decreases the film thickness at the gas–liquid interface by turbulent shear [20]. In this case the k_La was calculated for colloidal chitin in medium 5 (M5CC) and SDG waste in medium 5 (M5SDG), which was compared to water. This was to compare the mass transfer resistance at different agitation rates and to understand the effect of different media constituents toward the k_La.

From Table 2, k_La values showed a consistent increase with agitation and aeration rates. It was nevertheless interesting to note the wide difference of k_La in M5SDG and in distilled water at similar agitation and aeration rates. It is also suggested that M5SDG contained a wide mixture of salts and buffer, as well as the shrimp waste that were high in other complex nutrients and minerals. The difference showed that dissolved salts indeed influenced k_La of the media that resonated with the investigation by Schumpe [42] that showed that the decreased in k_La was proportional to the salt concentration in the media. Similar report on k_La was described by Felse and Panda [18], but their k_La results were much higher than those reported in this study.

Essentially, the oxygen uptake rate for *T. virens* UKM1 at optimal conditions in M5SDG during the highest chitinase productivity was 1.74 mg of DO per gram of fungal biomass per hour, which was considerably lower than the corresponding k_La of 8.34 per hour, suggesting no DO limitations even though low flow rate was employed.

Conclusion

The best agitation and aeration rates using colloidal chitin as control substrate for optimized M5 were 200 rpm and 0.33 vvm with 4.1 U/l per hour and 5.97 U/l per hour of maximum volumetric chitinase activity, respectively. *T. virens* UKM1 was found to be shear sensitive from microscopic observations. The k_La using SDG waste as substrate gave highest chitinase productivity of 1.74 mg of DO per gram of fungal biomass per hour at 8.34 per hour. Essentially there was no DO limitations during the highest chitinase production.

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