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Optimization of fresh palm oil mill effluent biodegradation with *Aspergillus niger* and *Trichoderma virens*

Noorbaizura Jalaludin[1], Roshanida A. Rahman[1]*, Firdausi Razali[1], Hind F.A Barghash[2], Siti Sabrina Mohd Sukri[1]

[1]Universiti Teknologi, Malaysia
Faculty of Chemical Engineering
[2]College of Applied Science, Oman

*Corresponding author's e-mail: roshanida@cheme.utm

Abstract: In this work, response surface optimization strategy was employed to enhance the biodegradation process of fresh palm oil mill effluent (POME) by *Aspergillus niger* and *Trichoderma virens*. A central composite design (CCD) combined with response surface methodology (RSM) were employed to study the effects of three independent variables: inoculum size (%), agitation rate (rpm) and temperature (°C) on the biodegradation processes and production of biosolids enriched with fungal biomass protein. The results achieved using *A. niger* were compared to those obtained using *T. virens*. The optimal conditions for the biodegradation processes in terms of total suspended solids (TSS), turbidity, chemical oxygen demand (COD), specific resistance to filtration (SRF) and production of biosolids enriched with fungal biomass protein in fresh POME treated with *A. niger* and *T. virens* have been predicted by multiple response optimization and verified experimentally at 19% (v/v) inoculum size, 100 rpm, 30.2°C and 5% (v/v) inoculum size, 100 rpm, 33.3°C respectively. As disclosed by ANOVA and response surface plots, the effects of inoculum size and agitation rate on fresh POME treatment process by both fungal strains were significant.

Introduction

Malaysia is known as the largest producer and exporter of palm oil and palm oil products (Vijayaraghavan et al. 2007). However, the production of palm oil leads to the increasing amount of polluted effluent namely palm oil mill effluent (POME) that possesses extremely high biological and chemical oxygen demands (BOD and COD) that cause great threat to the quality of water environment nearby (Zhang et al. 2008a). 44 million tons of POME was generated during the year of 2008 and if discharged untreated, this would then make the palm oil mill industry in Malaysia an industry which generates the biggest pollution load into the drainages (Wu et al. 2010). In order to keep the economy growing, besides preserving the environment, an effective and environmental-friendly method regarding POME management in palm oil mills is vital.

Consequently, the most common practice adopted by most mills for treating raw POME is ponding or open digesting tank system. Unfortunately, this treatment method has several drawbacks, such as the demand for large space of area, long hydraulic retention time (HRT) of 40–60 days, and production of corrosive and odorous gas directly to the atmosphere which could have detrimental impacts to the environment (Wu et al. 2010). Moreover, this commonly used conventional system sometimes failed to meet the standard discharge limits set by the Department of Environment (DOE) (Bhatia et al. 2007).

Additionally, existing techniques for POME remediation including treatment of POME using integrated anaerobic--aerobic bioreactor (IAAB) (Chan et al. 2012), upflow anaerobic sludge bioreactor (UASB) (Ahmad et al. 2011) and anaerobic bench scale reactor (ABSR) (Tjoon Tow Teng et al. 2013) demand high capital and operating costs.

Filamentous fungi have been recently employed in the treatment of domestic and industrial wastewater because of their capability to break down and transform the organic and toxic pollutants into disposal waste (Fakhru'l-Razi and Molla 2007, Liu et al. 2011). Additionally, utilization of fungi in terms of bioseparation and dewaterability in wastewater has been well documented in the literature (Alam et al. 2003, Mannan et al. 2005). Besides fungi, earthworms have also been utilized as a biodegrader of POME (Su Lin Lim et al. 2014). Furthermore, their potential in recovering fungal biomass protein had been discovered in several works as well (Jin et al. 1998, Zhang et al. 2008b). Although the application of filamentous fungi for wastewater treatment has been extensively studied, there are only a few published articles on POME bioremediation.

Prior to the utilization of filamentous fungi for the bioremediation of contaminated wastewater, further study on the physical parameters that affect the biodegradation is essential. Adequate experimental design is vital to determine the effect of the process parameters on the biodegradation process. Thus, a statistically designed experimental protocol

known as response surface methodology (RSM) could be employed rather than using 'one-factor-at-a-time' technique which needs a significant amount of work and time (Lee et al. 2012, Alam et al. 2007).

Thus, the objective of this present study is to optimize the fresh POME biodegradation by *A. niger* and *T. virens* so that better quality of POME with maximum production of biosolids enriched with fungal biomass and protein could be achieved. Central composite design (CCD) was employed to determine the optimal conditions which might enhance the fresh POME biodegradation in terms of TSS, COD, turbidity, SRF, biosolids enriched with fungal biomass and protein yield.

Materials and methods

Sample collection and preparation
Freshly discharged POME was collected from PPNJ (Persatuan Peladang Negeri Johor) Palm Oil Mill Factory in Kahang, Johor, Malaysia. The samples were preserved at a temperature of 4°C in cold room for immediate use. The characteristic of POME that was used in the experiment is shown in Table 1.

Inoculum Preparation
Two different fungi *A. niger* (ATCC120120) and *T. virens* (UKM-1) were isolated from the stock culture of Bioprocess Engineering Laboratory, Faculty of Chemical Engineering, Universiti Teknologi Malaysia and were used as inoculum throughout the study. The cultures were grown on PDA plates and incubated for ten days at 30+2°C. Afterwards, cultures of the petri dishes were harvested with 0.01% Tween 80 sterilized solution and mycelial suspension obtained was centrifuged using refrigerated centrifuge followed by washing three times with sterile distilled water in order to completely remove the Tween 80. For each type of fungi, its concentration (spores/ml) had to be measured by using Haemocytometer prior to use.

Experimental design and procedure
A central composite design (CCD) combined with response surface methodology (RSM) was applied to determine the optimum operating conditions for treatment process of fresh POME with *A. niger* and *T. virens*. Table 2 shows the experimental range and levels of independent process variables considered in this study. Three independent variables with three coded levels were chosen as the most important process parameters based on the preliminary results.

Design Expert software version 6.04 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for the experimental design, data analysis and graphical analysis of the data. The design matrix of the CCD and the experimental results for both treatments (treatment of fresh POME with *A. niger* and *T. virens*) are presented in Table 3. Fungal treatment of fresh POME was conducted by performing the experiment in 500 ml Erlenmeyer flasks containing 250 ml of fresh POME. The fresh POME was autoclaved at 121°C for 45 min to prevent growth of other microorganisms and inoculated with various doses of inoculum size. The fresh POME inoculated with *A. niger* and T. virens were incubated in a rotary shaker for 2 days and 4 days respectively under various conditions of temperature and agitation rate (Table 3).

Analytical methods
The supernatant of the fresh POME was collected for analysis. TSS and COD of the treated supernatant were measured according to the standard method described by American Public Health Association (APHA 2005). The turbidity in Nephelometric Turbidity Unit (NTU) was measured using HACH turbidity meter. Specific resistance to filtration (SRF) test according to the procedure reported by Alam et al. (2003) was used to determine the specific resistance of sludge to filtration or dewatering. Sampling of TSS, COD, and turbidity were done in triplicate.

Biosolids determination
Dry biosolids enriched with fungal biomass were collected by filtering the fermentation broth through filter paper (Whatman#1) followed by rinsing twice with deionised water and drying to constant mass at 105°C for 24 hours. Afterwards, the dried biosolids weight was determined.

Table 1. Charateristics of fresh POME used in this study

Parameters	Concentration (mg/L)
Total suspended solids (TSS)	17000
Chemical oxygen demand (COD)	37610
pH	3–4

* Unit for all parameter is mg/l except pH

Table 2. Experimental range and levels of independent process variables

Variables	Symbols	Coded Levels				
		-1.414	-1	0	+1	+1.414
Inoculum size (%)	X_1	1.89	5	12.5	20	23.11
Agitation rate (rpm)	X_2	89.64	100	125	150	160.36
Temperature (°C)	X_3	28.76	30	33	36	37.24

Table 3. Design matrix of CCD and the experimental results (responses) obtained for both treatments (fresh POME treated by *A. niger* and fresh POME treated by *T. virens*)

| Run[14] | Experimental Design | | | Results | | | | | | | | | | | |
| | (X_1) | (X_2) | (X_3) | *A. niger* | | | | | | *T. virens* | | | | | |
				TSS (mg/l)	Turbidity (NTU)	COD (mg/L)	Biosolids (g/L)	Protein (g/L)	SRFx10^{14} (m/kg)	TSS (mg/L)	Turbidity (NTU)	COD (mg/L)	Biosolids (g/L)	Protein (g/L)	SRFx10^{14} (m/kg)
1	+1	+1	−1	340	637.67	6560	5.02	3.47	8.96	226	138.75	3520	3.5	5.3	0.63
2	+1	−1	+1	820	401.67	4640	55.32	13.27	4.24	387	230	3700	4.23	3.66	0.75
3	−1	+1	+1	290	539.67	6026	7.13	4.84	9.77	256	145	3840	9.8	5.23	0.16
4	−1	−1	−1	100	22.33	2400	38.31	7.08	0.81	122	37.8	3440	14.144	3.36	3.45
5	−α	0	0	120	95.40	4240	9.69	4.03	1.23	150	60	3500	6.15	3.98	2.41
6	+α	0	0	180	498.67	5440	59.88	9.17	9.75	220	144.33	3360	6.64	4	0.95
7	0	−α	0	80	48.43	4640	42.75	12.87	2.78	324	138	4230	8.06	3.36	1.33
8	0	+α	0	878	630.33	3520	13.43	9.24	5.42	276	200	4195	7	3.61	4
9	0	0	−α	276	287.67	3680	5.89	4.52	2.84	140	140	3520	5.55	2.32	2.68
10	0	0	+α	909	557.33	6000	12.43	4.80	2.56	530	519.25	4000	11.02	5.4	5.5
11	0	0	0	281	385.67	2773	14.57	5.67	9.55	208	144.25	4150	8.91	3.55	3.93
12	0	0	0	204	251.67	2506.67	14.30	6.54	13.55	240	110	4300	8.04	3.4	4.65
13	0	0	0	262	286.33	2506.67	14.12	6.18	8.66	206	125	4200	8.77	3.2	3.7

TSS: Total suspended solids (mg/L); Turbidity (NTU); COD: Chemical Oxygen Demand (mg/L); Biosolids: Biosolids enriched with fungal biomass (g/L); Protein yield (g/L); SRFx10^{14} (m/kg)

Protein analysis

Lowry method (Lowry et al. 1951) is an often cited general use protein assay and was used to determine the concentration of protein using bovine serum albumin (BSA) as the reference standard.

Verification of the models

Further experimental work was conducted to validate the predicted results obtained in both treatments by RSM. The experiments were performed according to the optimized experimental conditions suggested by RSM. The experimental values obtained for both treatments were then compared to the predicted values proposed by the models to indicate the adequacy of the models.

Results and discussion

Statistical Analysis

The fresh POME inoculated with *A. niger* and *T. virens* was subsequently incubated in rotary shaker for 2 days and 4 days respectively under various conditions of temperature and agitation rate according to optimized experimental design. The design matrix in coded unit based on the experimental design generated from the software (Design Expert, Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, Version 6.0.4) with the response for treatment of POME with *A. niger* and *T. virens* was as displayed in Table 3. The regression equations for various responses along with respective ANOVA results are demonstrated in Table 4.

Terms with a probability value of less than 0.05 indicate that the model terms are significant. The models selected for all the responses for treatment of fresh POME with *A. niger* and *T. virens* were proven statistically significant with very low probability values (0.0328 to <0.0001) (Table 4). Additionally, high value of determination coefficient R^2 of closer to 1 signifies a good correlation between the experimental data and predicted data (Wang and Lu 2005). For instance, the determination of coefficient R^2 of 0.9997 for production of biosolids enriched with fungal biomass for treatment of fresh POME with *A. niger* was substantially high implying that 99.97% of the total variability of the response model could be described by the model. Furthermore, the 3-dimensional response surface and contour plots were constructed by the Design Expert software to investigate the interactions between the independent variables on the response process. Detailed analysis of these models via response surfaces and contour plots is presented in the following sections.

Analysis and comparison of treatment of fresh POME with A. niger and T. virens
Effect of inoculum size and agitation rate on TSS

The effect of agitation rate and inoculum size on the total suspended solids (TSS) in fresh POME treated with *A. niger* and *T. virens* as 3-D response surfaces and respective contour plots are shown in Fig. 1 (A) and (B) respectively. At lower agitation rate of 100 rpm, as shown in Fig. 1 (A), the TSS in fresh POME treated with *A. niger* was reduced. This is due to the fact that the settleability of the fresh POME was enhanced at lower agitation rate, where the solid particles trapped by the fungal strain were attached together forming bigger flocs/biosolids at the bottom of the treatment flask and solids

free supernatant was obtained. However, as the agitation rate was increased up to 150 rpm, the TSS increased to 659.45 mg/L since the strong mechanical forces caused by over-agitation might prevent the solids particles from being trapped by the fungal strain to form biosolids in fresh POME treated with *A. niger*.

On the contrary, Fig. 1 (B) represents the interaction effect between inoculum size and agitation rate on the TSS at a fixed temperature of 33°C. The saddle nature observed in Fig. 1 (B) implies that the interaction between inoculum size and agitation rate affects the TSS concentration in fresh POME treated with *T. virens* drastically. The TSS in fresh POME treated with *T. virens* drastically dropped to 97.09 mg/L with increasing agitation rate speed up to 150 rpm. This finding is in agreement with the study reported by Alam et al. (2003), which revealed that adopting agitation rate of 150 significantly increased the settleability process in fungal treated sludge with mixed culture of *A. niger* and *P. corylophilum* in a batch fermenter. Additionally, greater removal of TSS of up to 85% was achieved in treatment of domestic wastewater sludge by fungal entrapped biosolids using 150 rpm (Fakhru'l-Razi and Molla 2007).

In the meantime, a study reported by Mannan et al. (2005) disclosed that extension of treatment period might increase the soluble and insoluble biomass in the treatment which in turn might increase the TSS of the filtrate due to the effect of agitation rate when the sludge was treated with *A. niger* rather than *P. corylophilum*. Therefore, these findings show that different filamentous fungi and the intensity of mechanical forces on the mycelium might influence the treatment process and thus confirming the contrast of the results obtained between the treatment with *A. niger* and *T. virens* in the present study.

Agitation is vital for proper oxygen transfer and provides sufficient space for the cells to have access to all the substrates, however, increasing agitation intensity might increase the mechanical forces on the fungal cells, and therefore lead to reduction in cells growth due to shear stress (Fadzilah and Mashitah 2010). Thus, moderate agitation rate is the most conducive condition which could enhance biodegradation of the organic matter present in fresh POME by increasing the contact between the fungal cells and the substrate, and so will lead to greater reduction in TSS.

Effect of inoculum size and agitation rate on turbidity

The results of turbidity of fresh POME as affected by inoculum size and agitation rate for fresh POME treated with *A. niger* and *T. virens* in terms of response surfaces and corresponding contour plots are demonstrated in Fig. 2 (A) and (B) respectively. At fixed temperature of 33°C, the turbidity of fresh POME treated with *A. niger* rapidly increased to 685.13 NTU as the inoculum size and agitation rate increased to 20% and 150 rpm, respectively. Thus, this finding showed that low turbidity (29.15 NTU) was achieved at low dose of inoculum size (5%) and low speed of agitation (100 rpm) as displayed in Fig. 2 (A). Entrapment of the suspended solids by the fungal strain is the probable mechanism responsible for the decreasing trend of the turbidity (Liu et al. 2011). However, at low agitation rate (100 rpm), the reduction of turbidity in fresh POME treated with *A. niger* was significantly enhanced as compared to those using high agitation rate (150 rpm).

Table 4. Regression equations and ANOVA results for responses parameters for treatment of fresh POME by *A. niger* and *T. virens*

Responses	Regression equations in terms of coded factors	Prob>F	R²	Adj.R²	PLOF	CV	A.P	SD
A. niger								
TSS	$272.24 + 21.21 X_1 + 282.14 X_2 + 223.80 X_3 - 78.54 X_1^2 + 85.96 X_2^2 + 142.71 X_3^2 + 56.30 X_1X_2 + 354.64 X_1X_3 - 171.29 X_2X_3$	0.0150	0.9841	0.9365	0.0997	20.64	12.562	75.24
Turbidity	$357.14 + 130.96 X_1 + 197.03 X_2 + 82.84 X_3$	<0.0001	0.9199	0.8932	0.5830	19.52	17.827	69.72
COD	$2754.51 + 558.80 X_1 - 395.98 X_2 + 820.24 X_3 + 923.53 X_1^2 + 543.53 X_2^2 + 923.53 X_3^2 + 393.58 X_1X_2 - 1782.65 X_1X_3$	0.0098	0.9676	0.9028	0.0567	10.82	10.274	457.30
Biosolids	$14.78 + 15.69 X_1 - 10.37 X_2 + 2.29 X_3 + 8.41 X_1^2 + 6.51 X_2^2 - 2.97 X_3^2 - 2.49 X_1X_2 + 10.00 X_1X_3 + 11.96 X_2X_3$	<0.0001	0.9997	0.9989	0.0861	2.73	94.908	0.6
Protein yield	$6.35 + 1.82 X_1 - 1.28 X_2 + 0.099 X_3 + 0.038 X_1^2 + 2.19 X_2^2 - 1.01 X_3^2 - 1.79 X_1X_2 + 1.73 X_1X_3 + 0.61 X_2X_3$	0.0112	0.9870	0.9481	0.1279	10.34	15.464	0.73
SRF x 10¹⁴	$10.74 + 3.01 X_1 + 0.93 X_2 + 8.05 X_3 - 2.74 X_1^2 - 3.44 X_2^2 + 1.62 X_3^2 + 6.99 X_1X_2 - 2.49 X_1X_3 + 2.36 X_2X_3$	0.0328	0.9728	0.8914	0.7898	27.41	12.774	2.18
T. virens								
TSS	$250.80 + 41.75 X_1 - 11.86 X_2 + 137.89 X_3 - 35.96 X_1^2 + 39.04 X_3^2 + 64.14 X_1X_2$	0.0044	0.9210	0.8420	0.1214	17.41	12.513	44.00
Turbidity	$143.45 + 38.15 X_1 + 12.95 X_2 + 134.09 X_3 - 40.14 X_1^2 + 37.59 X_2^2 + 84.47 X_3^2$	0.0079	0.9020	0.8057	0.0717	31.61	12.379	51.86
COD	$4215.00 - 32.25 X_1 + 21.31 X_2 + 157.35 X_3 - 385 X_1^2 X_2^2 - 220.00 X_3^2$	<0.0001	0.9822	0.9695	0.9695	1.59	20.361	61.14
Biosolids	$8.85 + 1.61 X_1 + 0.70 X_2 + 2.04 X_3 + 1.28 X_1X_2 + 3.63 X_1X_3 + 4.29 X_2X_3$	0.0031	0.9302	0.8603	0.1236	13.63	13.588	1.07
Protein yield	$3.29 + 0.05 X_1 + 0.088 X_2 + 1.09 X_3 + 0.42 X_1^2 + 0.17 X_2^2 + 0.36 X_3^2 + 1.03 X_1X_2 - 0.79 X_1X_3$	0.0092	0.9687	0.9060	0.1954	7.25	13.180	0.28
SRFx10¹⁴	$4.31 - 0.54 X_1 + 0.94 X_2 + 1.00 X_3 - 1.48 X_1^2 - 0.99 X_2^2 - 0.27 X_3^2 + 1.79 X_1X_2 + 1.80 X_1X_3$	0.0091	0.9386	0.8525	0.3147	25.29	9.680	0.66

This is because the bioseparation process at low agitation rate was enhanced in fresh POME treated with *A. niger* as the suspended solids were trapped by the fungal strains forming bigger biosolids/flocs and solids free supernatant was released. On the other hand, inclination of turbidity at high agitation rate was largely due to shearing effects caused by over-agitation on the fungal strain therefore preventing accumulation of biosolids. With regard to inoculum size, optimal inoculum size is crucial for biodegradation process since overcrowding of spores may lead to depletion of nutrients and inhibit growth and development (Yesilada *et al.* 1998).

Meanwhile, Fig. 2 (B) shows the effect of inoculum size and agitation rate on the turbidity of fresh POME treated with *T. virens* at a fixed temperature of 33°C. Similar to Fig. 1 (B), the response surface and contour plots show a surface with a saddle point. High bioseparation (low turbidity) was achieved at high agitation rate (150 rpm) which agrees with the finding obtained by Mannan et al. (2005) in sludge treated with *Penicillium corylophilum*. Greater removal of turbidity (98.91%) was observed at agitation rate of 150 rpm in the sludge treated using *P. corylophilum*. Additionally, these findings revealed the strong influence of the TSS on the turbidity of fresh POME during the treatment period in which reduction of TSS could result in the reduction of turbidity as well.

Effect of inoculum size and agitation rate on chemical oxygen demand (COD)

The estimation of COD in fresh POME treated with *A. niger* and *T. virens* with regard to the independent variables of inoculum size and agitation rate at fixed temperature in terms of response surfaces and respective contour plots are illustrated in Fig. 3 (A) and (B) respectively. In Fig. 3 (A), the COD of fresh POME treated with *A. niger* decreased when inoculum size and agitation rate increased up to a certain level and further decreased with increments of agitation rate of up to 150 rpm. Similarly, Yesilada et al. (1998) also observed a better removal of COD when the agitation speed between 150–200 rev/min was adopted in the treatment of olive oil mill wastewater (OMW) with *Coriolus versicolor* and *Funalia trogii*. Therefore, these findings signify that better degradation of organic compounds could be achieved at high rate of agitation due to the increase of dissolved oxygen level in the treatment process. Furthermore, low COD was favored at intermediate dose of inoculum size 8.75–12.50% (v/v) in fresh POME treated with *A. niger*. Similarly, a study by Truong *et al*. (2004) showed that maximum COD removal of 85.5% was achieved by adopting 10% dose of *A. niger* compared to 1% (v/v) (75% COD removal) from cassava starch processing wastewater, hence proving that COD removal rate is relatively better within the doses mentioned which is 10%.

A
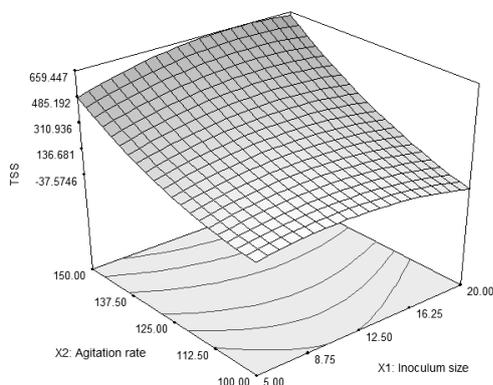
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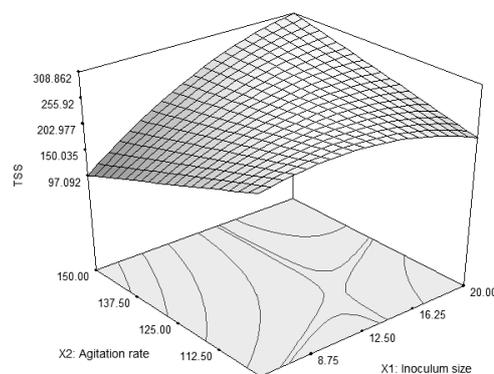
Fig. 1. 3-dimensional response surface of TSS representing the effect of agitation rate and inoculum size: (A) fresh POME treated by *A. niger* (B) fresh POME treated by *T. virens*

A
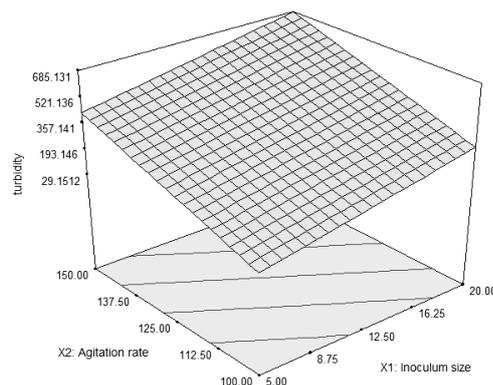
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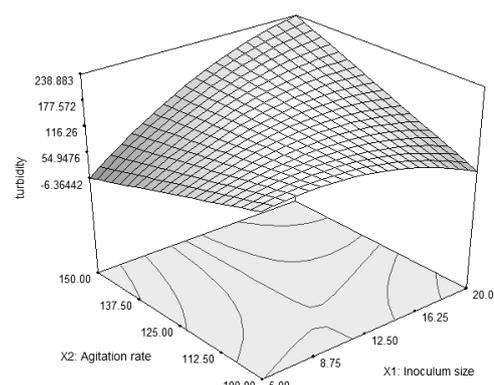
Fig. 2. 3-dimensional response surface of turbidity representing the effect of agitation rate and inoculum size: (A) fresh POME treated by *A. niger* (B) fresh POME treated by *T. virens*

On the contrary, Fig. 3 (B) displays the mutual effect of inoculum size and agitation rate at optimal temperature of 33°C on the COD in fresh POME treated with *T. virens*. Lower and high dose of inoculum of 5% and 20% produced a lower value of COD. On the other hand, the flat response displayed along the agitation rate axis indicated that the effects of the variable on COD of treated fresh POME with *T. virens* was negligible.

Effect of inoculum size and agitation rate on biosolids enriched with fungal biomass

Fig. 4 (A) illustrates the 3-dimensional response surface and corresponding contour plots of the effect of inoculum size and agitation rate on the production of biosolids enriched with fungal biomass in fresh POME treated with *A. niger*. As the inoculum size was increased to 20%, the production of biosolids rapidly increased up to 58.24 g/L. On the other hand, at high agitation rate, the production of biosolids decreased to 4.9 g/L. High agitation rate prevents fungal cells from using the nutrient available and transferring the oxygen because of the mechanical forces caused by the strong agitation rate (Sepehr et al. 2005). The findings support the notion of both inoculum size and agitation rate as the critical factors influencing the production of biosolids enriched with fungal biomass in fresh POME treated with *A. niger*. Additionally,

these findings are also in agreement with the investigation done by Petre et al. (2010) revealing that the highest yield of fungal biomass (3.28+0.15 g/l) was achieved by adopting 12% (v/v) of inoculum size as compared to 2% (v/v) (2.95+0.23 g/l) in submerged fermentation of *Cordyceps sinensis*.

On the other hand, the production of biosolids enriched with fungal biomass in fresh POME treated with *T. virens* was in contrast with the treatment with *A. niger*. Noticeably, the production of biosolids declined as the inoculum size and agitation rate increased (Fig 4 (B)). This finding reveals that by adopting low speed of agitation and adding low dose of inoculum a significant amount of biosolids enriched with fungal biomass could be produced in fresh POME treated with *T. virens*.

Effect of inoculum size and agitation rate on protein yield

Fig. 5 (A) and (B) show the mutual effect of inoculum size and agitation rate on the yield of protein in biosolids in fresh POME treated with *A. niger* and *T. virens*, respectively, at optimal temperature of 33°C. As noted in Fig. 5 (A), the enriched protein in biosolids in fresh POME treated with *A. niger* increased rapidly with the increase of inoculum size to 20%. High protein yield of up to 13.39 g/L in biosolids for fresh POME treated with *A. niger* was contributed by high

A

B
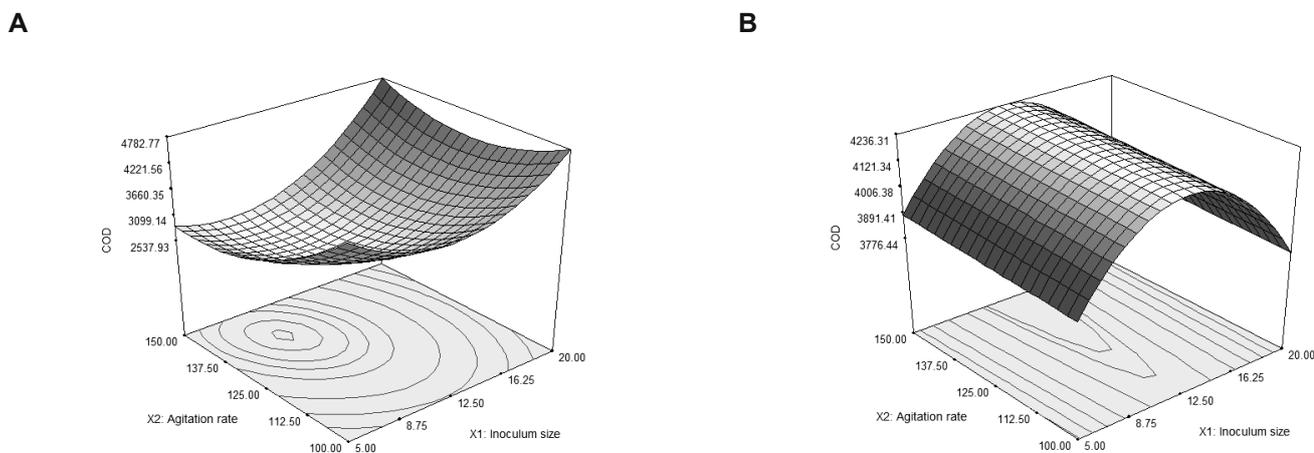
Fig. 3. 3-dimensional response surface of COD representing the effect of agitation rate and inoculum size: (A) fresh POME treated by *A. niger* (B) fresh POME treated by *T. Virens*

A

B
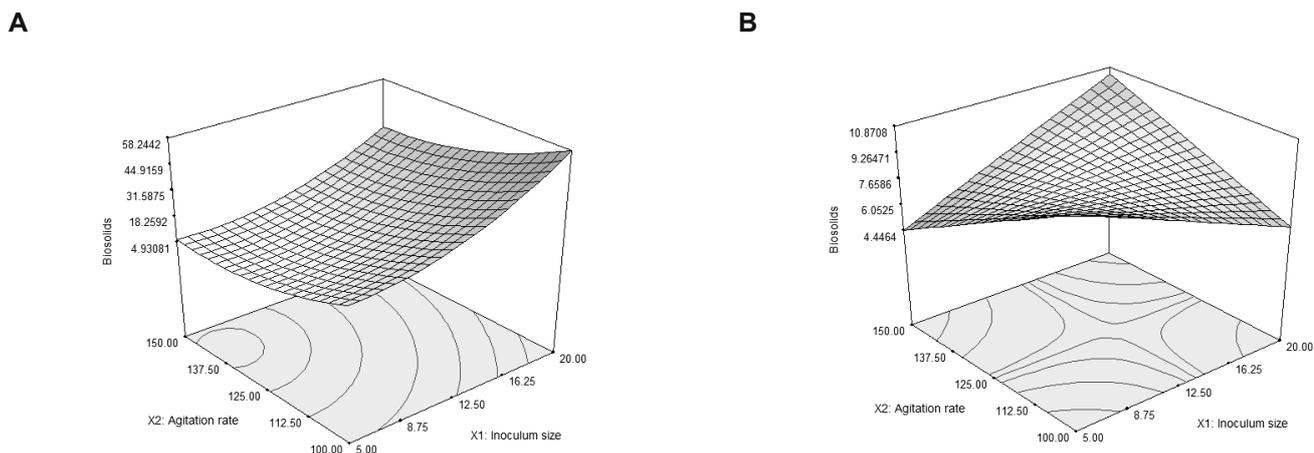
Fig. 4. 3-dimensional response surface of biosolids enriched with fungal biomass representing the effect of agitation rate and inoculum size: (A) fresh POME treated by *A. niger* (B) fresh POME treated by *T. Virens*

dose of inoculums (20%) which is in agreement with the finding obtained by Jin *et al.* (1998) who stated that inoculum size of 7.5% (v/v) was more favorable in synthesizing microbial biomass protein with 26.7% higher as compared to 1% (v/v). The effect of agitation rate on protein yield in biosolids was less apparent as compared to inoculum size. Since the inoculum size has a lower probability value (Prob>F: 0.0155) in the linear terms of the quadratic equation than the agitation rate (Prob>F: 0.0390), this proves that the influence of inoculum size on protein yield in biosolids for fresh POME treated with *A. niger* is far greater as compared to agitation rate.

On the contrary, a drastic fall in protein yield was observed in fresh POME treated with *T. virens* (Fig. 5 (B)) as the inoculum size and agitation rate were increased to 20% and 150 rpm. High yield of protein (5.05 g/L) could be achieved by adopting low speed of agitation (100 rpm) and adding small dose of inoculums (5%) in the treatment process. The interactions between inoculum size and agitation rate on protein yield in fresh POME treated with *A. niger* and *T. virens* were similar to those between inoculum size and agitation rate on the production of biosolids enriched with fungal biomass thus revealing the strong influence of the production of biosolids enriched with fungal biomass on the protein concentration obtained.

Effect of inoculum size and agitation rate on specific resistance to filtration (SRF)

The effect of inoculum size and agitation rate as well as their interaction on SRF at fixed temperature of 33ºC in fresh POME treated with *A. niger* and *T. virens* as 3-dimensional response surface plots and corresponding contour plots are presented in Fig. 6 (A) and (B) respectively. As the inoculum size was increased to 20%, the SRF rapidly decreased in both treatment processes (Fig 6 (A) and (B)). Similarly, drastic falls in SRF were observed when the agitation rates were increased beyond 100 rpm (Fig 6 (A) and (B)). These findings signify that by adopting high dose of inoculums (20%) and high speed of agitation (150 rpm), low SRF could be achieved in both treatment processes.

Similarly, better dewaterability (99.8% (1% w/w TSS)) was also achieved in domestic wastewater sludge treated using mixed culture of *Aspergillus niger* and *Penicillium corylophilum* when adopting agitation speed of 150 rpm. Moreover, lowest SRF (1.4×10^{12} m/kg) value for filtration was observed in treated sludge after 6 days of treatment using batch fermenter at an agitation rate of 150 rpm (Alam et al. 2003). Agitation rate >100 rpm is suitable to enhance the dewaterability/filterability of the treated wastewater as the solid particles are entrapped and compressed by the filamentous fungi and thereby accelerating the filtration process.

A

B
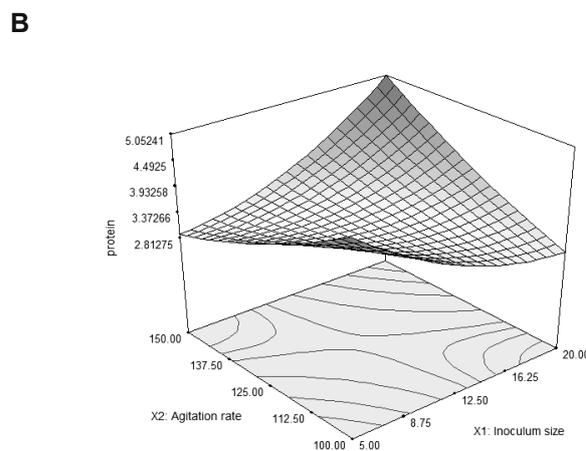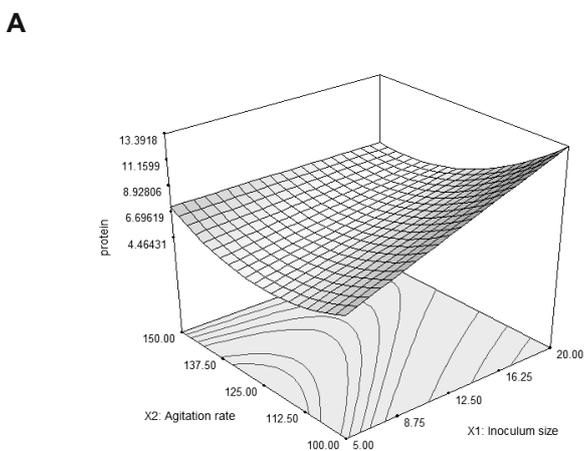
Fig. 5. 3-dimensional response surface of protein yield in biosolids representing the effect of agitation rate and inoculum size: (A) fresh POME treated by *A. niger* (B) fresh POME treated by *T. Virens*

A

B
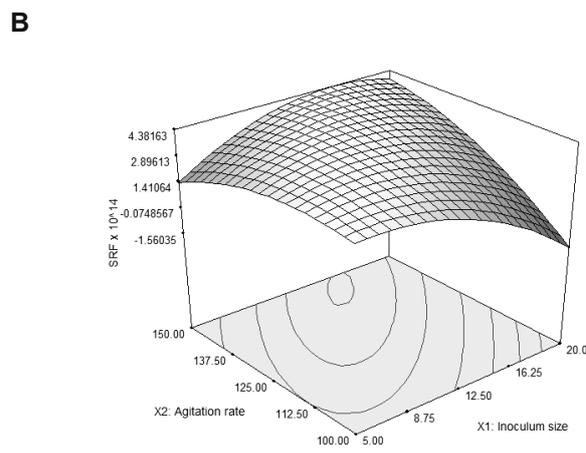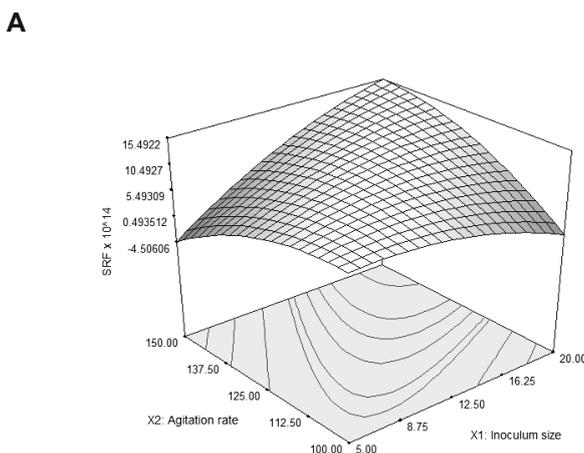
Fig. 6. 3-dimensional response surface of SRF representing the effect of agitation rate and inoculum size: (A) fresh POME treated by *A. niger* (B) fresh POME treated by *T. virens*

Optimization point prediction and verification

Following the design and analysis of the experimental data, numerical optimization was carried out for both treatments of fresh POME (*A. niger* and *T. virens*). The criteria of the variables were set accordingly and the responses such as TSS, COD, turbidity and SRF were set to minimum and the production of biosolids enriched with fungal biomass and protein yield was set to maximum. Table 5 depicts the optimum experimental conditions for fresh POME treated with *A. niger* and *T. virens* suggested by the RSM respectively.

Further experimental work was conducted to validate the predicted results obtained by RSM. The experiments were performed according to the suggested experimental conditions. The adequacy of the model for both treatments in predicting the optimum results for each response were proven by the experimental data obtained as listed in Table 6.

The data in Table 6 revealed that minimum values of TSS and SRF were achieved in fresh POME treated with *A. niger* and minimum values of turbidity and COD were achieved in fresh POME treated with T. virens. Though TSS value in fresh POME treated with *A. niger* was much smaller compared to *T. virens* yet the turbidity and COD were slightly higher as compared to those obtained in fresh POME treated with *T. virens*. This is probably due to the addition of high dose of

inoculum size (19% (v/v)) in the treatment process which leads to excessive spores in fresh POME. Therefore, high turbidity and COD were obtained due to the high organic content in the supernatant. Nevertheless, greater production of biosolids enriched with fungal biomass and protein was achieved in fresh POME treated with *A. niger* as compared to *T. virens*. Yet, based on the predicted and experimental results presented, the experimental values for both treatment processes (fresh POME treated with *A. niger* and *T. virens*) are in good agreement with the predicted values proposed by RSM with error of less than 10%.

Evaluation of Substrate Utilization and Cell Growth

Figs 7 and 8 present a plot of COD and VSS concentration versus time in batch treatment of fresh POME treated with *A. niger* and *T. virens* respectively. As can be seen in Fig. 7, germination of spores and growth of *A. niger* started 48 hours after inoculation in which it took about 48 hours for *A. niger* to adapt with new environment with no change of the cell number. Meanwhile, the exponential growth phase extended from about 48 hours to 96 hours after incubation. Noticeably, the substrates component which was expressed in terms of COD was initially larger at the beginning of the treatment; however it started to decrease over time. The exhaustion of

Table 5. Optimum experimental conditions for fresh POME treated by *A. niger* and *T. virens*

Optimum conditions	A. niger	T. virens
Inoculum size (%)	19.38	5
Agitation rate (rpm)	100	100
Temperature (^{o}C)	33.33	30.15

Table 6. Verification experiments at optimum process conditions

Responses	Predicted	Experimental	Predicted Error (%)
A. niger			
TSS (mg/L)	58.90	62	5.00
Turbidity (NTU)	289.38	301.52	4.03
COD (mg/L)	4544.22	4400	3.17
Biosolids enriched with fungal biomass (g/L)	55.32	50.40	8.89
Protein yield (g/L)	13.21	12.9	2.35
SRF x 10^{14} (m/kg)	0.81	0.87	6.89
T. virens			
TSS (mg/L)	153.35	163	5.92
Turbidity (NTU)	75.70	79	4.18
COD (mg/L)	3493.11	3450	1.23
Biosolids enriched with fungal biomass (g/L)	13.90	12.7	8.63
Protein yield (g/L)	3.32	3.18	4.15
SRF x 10^{14} (m/kg)	3.85	4.10	6.09
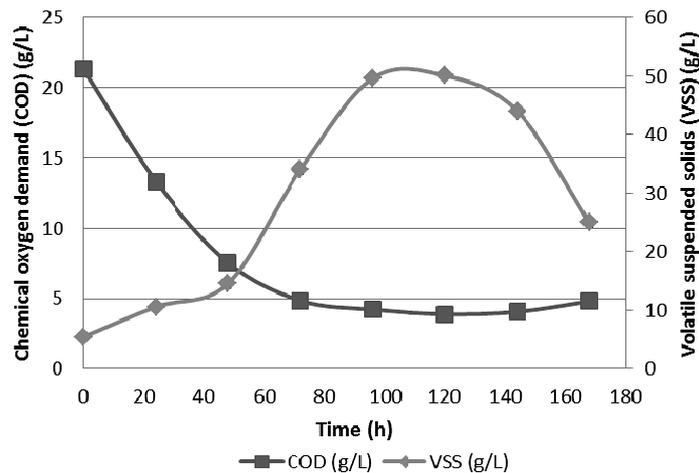
Fig. 7. COD and VSS concentration profiles in batch treatment of fresh POME by *A. niger*
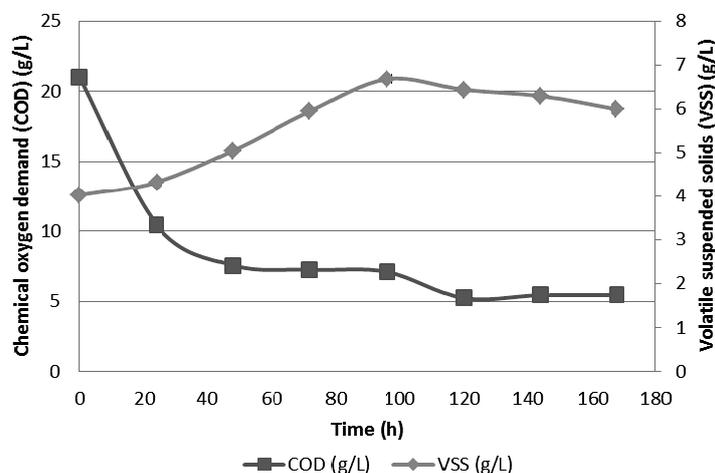
Fig. 8. COD and VSS concentration profiles in batch treatment of fresh POME by *T. virens*

the substrates components was due to consumption by *A. niger* for cell growth. Eventually, in the stationary phase which started at 96 hours and ended at 140 hours, the cell growth stopped since the essential nutrients had been depleted over time.

It has also been observed that the germination of spores and growth of *T. virens* started at 25 hours after inoculation. Based on Fig. 8, it can be seen that the exponential growth phase extended from about 25 hours to 85 hours after incubation. By contrast, the exponential growth exhibited by *T. virens* was less apparent to the exponential growth displayed by *A. niger* in which rapid increment of biomass can be seen in the exponential phase of treatment of fresh POME with *A. niger* than *T. virens*.

Summary

Implementation of filamentous fungi for POME treatment could help ease the maintenance, monitoring and land requirement entailed by the conventional ponding system. Since typical ponding system requires longer hydraulic retention time which involves higher operating costs, hence, by applying filamentous fungi to treat POME it is believed that the retention time required for treatment can be considerably

reduced. Moreover, it offers a better quality final effluent, less usage of chemicals, low energy consumption and low maintenance and operating costs since filamentous fungi can be easily obtained in nature.

Treatment of POME does generate greater amount of biosolids/sludge thus proper management of the biosolids is vital in order to prevent loss of effective treatment capacity as well as to alleviate public health risks due to unsafe disposal. Since POME is non-toxic, reuse of the biosolids for various applications is considered to be safe and would also be beneficial in terms of operating cost. Moreover, POME is rich in high biodegradable content of various suspended components of complex compounds such as proteins, fats, starch, cellulose, hemicelluloses, free organic acids and minor organic and mineral elements making them ideal substrates for production of fungal biomass protein. The fungal biomass contains substantial quantities of nutrients, such as carbohydrates, lipids, minerals and proteins thus making them also advantageous to be used as aquaculture feed ingredients. Furthermore, due to its high content of proteins and fibres, fungal biomass could possibly be applied in the food sectors as human and animal foodstuffs replacing traditional plant or animal sources (Moore and Chiu 2001).

Conclusion

The treatment of fresh POME with *A. niger* and *T. virens* using optimization by experimental design allowed us to determine the optimal conditions of the treatment processes. The findings achieved revealed that RSM is a feasible method for comparing the treatment process of fresh POME with two different fungal strains (*A. niger* and *T. virens*). As disclosed by ANOVA and response surface plots, the effects of inoculum size and agitation rate on fresh POME treatment processes with both fungal strains were significant, yet the treatment process of fresh POME with *A. niger* was shown to be more predictable and able to reduce greater TSS and SRF and also produce greater biosolids enriched with fungal biomass and protein as compared to *T. virens*.

Acknowledgments

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