

PRODUCTION AND STABILITY OF MYCO-FLOCCULANTS FROM *LENTINUS SQUARROSULUS* RWF5 AND *SIMPLICILLIUM OBCLAVATUM* RWF6 FOR REDUCTION OF WATER TURBIDITY

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ABSTRACT: The production and stability of two novel myco-flocculants produced by river water fungus (RWF) were investigated. Screening tests were conducted to find suitable nutrients, pH, nutrient concentration, inoculum dose, and stability for two myco-flocculants *L. squarrosulus* (RWF5) and *S. obclavatum* (RWF6). The strains showed good flocculating activity in reducing turbidity of kaolin suspension while malt extract was used as nutrient source. Supernatants of RWF5 and RWF6 were able to reduce turbidity from 900±10 NTU to 46 NTU (95%) and 195 NTU (78%), respectively. In order to enhance the production, optimization of cultivation conditions were studied using a one-factor-at-a-time (OFAT) method. *L. squarrosulus* (RWF5) reduced 96% of turbidity at optimum conditions, comprising of 0.1% (w/v) malt extract, 3% (v/v) inoculum dose, and initial pH 7.0 for 6 days. The results of the compatible mixed culture showed good flocculation activity at 88% compared to a single culture of *S. obclavatum* at 78%. On the other hand, *L. squarrosulus* showed better turbidity reduction in the single culture rather than the mixed culture. The stability of *L. squarrosulus* and *S. obclavatum* supernatants showed excellent turbidity reduction over a wide pH range of 4-8 with the maximal flocculation rate of 96% and 90%, respectively, at pH 7.0. They also exhibited high turbidity removal ability in a temperature range of 4 °C – 55 °C for 24h with a maximum turbidity removal rate of 96% (RW5) and 87% (RW6) at 25 °C. Time stability of the *L. squarrosulus* supernatant showed good turbidity removal potential at above 90% at room temperature (28± 2 °C) and 85% at low temperature (4 °C) for 12 days. The high flocculating rate of the myco-flocculants and their good stability under wide range of temperature indicated their potentiality as biodegradable flocculants for water and wastewater treatment industry.

ABSTRAK: Keberhasilan dan kestabilan dua myco-gumpalan baharu oleh kulat air sungai (RWF) telah dikaji. Ujian penapisan telah dijalankan untuk mencari nutrien sesuai, pH, kepekatan nutrien, dos inokulum dan kestabilan dua myco-gumpalan *L. squarrosulus* (RWF5) dan *S. obclavatum* (RWF6). Rangkaian menunjukkan aktiviti gumpalan yang baik dalam mengurangkan kekeruhan air sungai dengan penggantungan koalin di mana ekstrak malt telah digunakan sebagai sumber nutrien. Larutan supernatan RWF5 dan RWF6, masing-masing mampu mengurangkan kekeruhan dari 900±10 NTU kepada 46 NTU (95%) dan 195 NTU (78%). Bagi meningkatkan pengeluaran, keadaan optimum bagi menggalakkan hasil telah diselidiki menggunakan kaedah Satu Faktor pada Tiap Masa (OFAT). Pada takat optimum, *L. squarrosulus* (RWF5) mengurangkan 96% kekeruhan,

ianya terdiri daripada ekstrak malt 0.1% (w/v), dos inokulum 3% (v/v) dan pH awal 7.0 selama 6 hari. Keputusan kultur campuran yang sesuai menunjukkan aktiviti penggumpalan yang baik pada 88% berbanding kultur sendirian *S. obclavatum* pada 78%. Pada waktu sama, *L. squarrosulus* menunjukkan pengurangan kekeruhan yang lebih baik dalam kultur sendirian berbanding kultur campuran. Kestabilan larutan supernatan *L. squarrosulus* dan *S. obclavatum* menunjukkan pengurangan kekeruhan yang sangat baik pada pH yang luas iaitu 4-8 dengan kadar maksimum kekeruhan pada 96% dan 90%, pada pH 7.0 masing-masing. Keduanya menunjukkan kebolehan penyingkiran kekeruhan yang tinggi pada skala suhu 4 °C – 55 °C selama 24 jam dengan kadar nyah kekeruhan maksimum pada 96% (RW5) dan 87% (RW6) pada suhu 25 °C. Kestabilan masa larutan supernatan *L. Squarrosulus* menunjukkan potensi penyingkiran kekeruhan terbaik atas 90% pada suhu bilik (28± 2 °C) dan 85% pada suhu rendah (4 °C) selama 12 hari. Kadar gumpalan yang tinggi oleh myro-gumpalan dan kestabilan yang baik pada julat suhu yang luas menunjukkan potensinya sebagai agen biodegradasi gumpalan kepada air dan industri rawatan loji air.

KEYWORDS: *flocculation; jar test; Lentinus squarrosulu; mixed culture; Simplicillium obclavatu; stability; turbidity*

1. INTRODUCTION

Chemical flocculants are widely used in wastewater and drinking water treatment, food and fermentation industries, as well as in downstream processing due to their high flocculating efficiency and cost-effectiveness. [1, 2] Generally, flocculating agents that have the most usage in water treatment are the synthetic ones such as aluminium sulphate, ferric chloride, lime, and synthetic polymers. [3, 4] Aluminium salts are the most widely used coagulant in water and wastewater treatment because of their proven performance and cost effectiveness. However, chemical coagulants cannot be easily degraded in nature and may result in various health and environmental problems. [5] Compared with chemical flocculants, bioflocculants have more advantages, such as being environmentally friendly due to their biodegradability, largely nontoxic with no secondary pollution, and harmless to both humans and animals. [6] Therefore, bioflocculants have recently gained global attention in research because they hold immense potential in replacing chemical flocculants. For example, Li et al. [7] reported that *B. licheniformis* produced ZS-7 bioflocculant is expected to be a potential replacement for conventional synthetic flocculants such as PAC (polyaluminum chloride) and PAM (polyacrylamide) for the treatment of low temperature and room temperature (25 °C) drinking water. Similarly, Gong et al. [8] reported *Serratia ficaria* produced bioflocculant SF-1 showed 91.8 – 93.7% turbidity removal from wastewater, which is better than that of PAC and PAM. *Aspergillus flavus* produced IH-7 bioflocculant showed better flocculation performance than PAC and IH-7 was significantly used to flocculate different types of suspended solids such as activated carbons, kaolin clays, soil solids, and yeast cells [9]. Moreover, bioflocculants are widely used for the recovery of suspended solids (SS) from wastewater treatment [10-12].

Bioflocculants are secondary metabolites produced during the growth of microorganisms such as bacteria, yeast and fungi that are composed of polysaccharides, proteins, lipids, glycoproteins, and glycolipids [13-15]. The composition and properties of coagulants depend on the type of flocculant-producing microorganisms (BPMs), composition of the media, and many conditions. The differences in the composition and properties of polysaccharides and proteins lead to differences in the charge of coagulant [16]. Culture conditions screening is a powerful approach to increase the production of extra polymeric substances from microorganism cultivation [17]. Although most flocculants can

be used to flocculate kaolin suspension, they show different flocculating ability for other particles or colloids in aqueous solutions. Jebun et al. [18, 19] reported some filamentous fungi isolated from river water samples showed good entrapment potentiality to reduce turbidity from river water. Deng et al. [20] reported *Aspergillus parasiticus* produced a coagulant with high flocculating activity for kaolin suspension and water-soluble dyes. Pu et al. [21] reported that the compound coagulant produced by two strains of *Rhizopus sp.* was successfully used to reduce turbidity from potato starch wastewater. The same observation was made by Luvuyo et al. [22] for turbidity removal from kaolin clay by bioflocculant produced by a mixed culture of *Methylobacterium sp.* and *Actinobacterium sp.*

In the present study, we examine the suitable culture conditions for the production of myco-flocculants by two novel *L. squarrosulus* RWF5 and *S. obclavatum* RWF6 for reducing turbidity from synthetic turbid water. The screening tests were conducted to find suitable nutrients, pH, nutrients concentration and inoculum size of these two potential fungi to enhance flocculation performance. In addition, stability of the single and compatible mixed culture supernatants (myco-flocculants) was investigated for chemical and environmental conditions to reduce turbidity by jar test.

2. MATERIALS AND METHODS

2.1 Microorganisms and Growth Conditions

L. squarrosulus RWF5 and *S. obclavatum* RWF6 were isolated from river water samples collected from the River Pusu on the IIUM campus for coagulant production. *L. squarrosulus* RWF5 and *S. obclavatum* RWF6 cultures were maintained on potato dextrose agar (PDA) (Oxoid, UK) and initial pH was adjusted to 5.8 ± 0.2 . Distilled water was used to prepare medium solutions and sterilized at $121\text{ }^{\circ}\text{C}$ for 20 minutes. The culture plates were incubated at $32 \pm 2\text{ }^{\circ}\text{C}$ for 10 days. Subculture was conducted twice in a month and sub culture plates were stored at $25\text{ }^{\circ}\text{C}$ in an incubator for further use.

2.2 Myco-coagulants Production

2.2.1 Screening of Nutrients

The screening medium consisted of 5 g/l starch, sucrose, yeast extract, and malt extract and each of the medium was mixed with 1 liter of distilled water. The medium was then sterilized by autoclaving it at $121\text{ }^{\circ}\text{C}$ for 15 minutes and then inoculated with 2% (v/v) fungal mycelial inoculum (340 mg/l). The liquid culture was then incubated in a rotary shaker with agitation at 150 rpm at $30 \pm 2\text{ }^{\circ}\text{C}$ for 8 days. Initial pH of the broth was adjusted at 7.0 ± 0.1 using 1 M NaOH or 2 M HCl. Supernatants were taken at different time intervals to determine the flocculation activity. The effect of cultivation time for myco-flocculants production was investigated for 8 days.

2.2.2 Factors Affecting the Flocculating Rate

To study the effect of malt extract concentration of 0-1% (g/l), initial pH values of 5, 6, 7, and 8, and inoculum doses of 1-4% on the flocculating rate were investigated. In this experiment, OFAT analysis was followed for three factors such as media concentration, pH, and inoculum dose.

2.2.3 Single and Mixed Culture

The production medium consisting of 5 g/l malt extract was autoclaved at $121\text{ }^{\circ}\text{C}$ for 15 minutes. The sterilized medium was inoculated with 2% (v/v) fungal inoculum for single

and mixed culture (mycelium concentration of 340 mg/l). Ten day old culture plates were used to prepare fungal inoculum and the culture was incubated in a rotary shaker with 150 rpm at 30 ± 2 °C for 6 days. Initial pH of the culture was adjusted at 7.0 ± 0.1 using 1 M NaOH or 2 M HCl. The cultures were harvested after 6 days of treatment and centrifuged to separate the biomass from supernatant (10^3 rpm for 10 mins at 25 °C). The supernatants were used as myco-flocculants and stored at room temperature at 28 ± 2 °C for further use.

2.3 Myco-flocculant Stability

2.3.1 pH Stability of Supernatants

The pH of myco-flocculants from single and mixed cultures was adjusted to 4, 5, 6, 7, and 8 with 1 M NaOH and 2 M HCl. A Jar test was conducted to observe flocculation activity in different pH. Initial turbidity and pH of kaolin suspension were recorded at 900 ± 10 NTU and 7.0 ± 0.1 , respectively and the mixture was stirred at 120 rpm for 40 minutes and allowed to settle for 30 minutes.

2.3.2 Temperature Stability

Myco-flocculants were treated at different temperatures of 4, 25, 35, 45 and 55 °C for 24 hours. Both supernatants were applied in kaolin suspension to reduce turbidity with the same conditions as referred to section 2.3.1.

2.3.3 Time Stability of *L. squarrosulus* Produced Myco-flocculant

The myco-flocculant produced by *L. squarrosulus* RWF5 was tested for flocculation activity capability. The myco-flocculant was kept in room temperature (28 ± 2 °C) and low temperature (4 °C) for 20 days. Jar tests were conducted to observe flocculation activity in terms of reducing turbidity from kaolin suspension.

2.3.3 Flocculation Activity

Kaolin suspension was prepared using 0.7 g kaolin clay in 1 liter of tap water (turbidity 900 ± 10 NTU). Each Jar contained 500 ml kaolin suspension was added with 1% supernatant (v/v). The jar apparatus was then operated at a speed of 120 rpm with 40 minutes mixing time and allowed to settle for 30 minutes [19]. Next, the top layer of water in each Jar was collected with a micro pipette and turbidity was measured with a portable turbidimeter 2100Q HACH, USA to measure residual turbidity. In the control experiment, 1% (v/v) of supernatant was replaced with 1% (v/v) of nutrient broth. The flocculating activity was calculated according to the following equation [23].

$$\text{Flocculating activity (\%)} = \left[\frac{A-B}{A} \right] \times 100\% \quad (1)$$

where, *A* is the initial turbidity value and *B* is the residual turbidity after flocculation.

3. RESULTS AND DISCUSSION

3.1 Myco-flocculant Production

3.1.1 Screening of Potential Nutrient

Figure 1 (a) shows the flocculating activity of two coagulants for 6 days in media containing starch, sucrose, yeast extract, and malt extract. Starch, sucrose, and yeast extract were not favourable for *L. squarrosulus* and *S. obclavatum* growth, while the production of coagulants was relatively low when carbon and nitrogen sources were used separately.

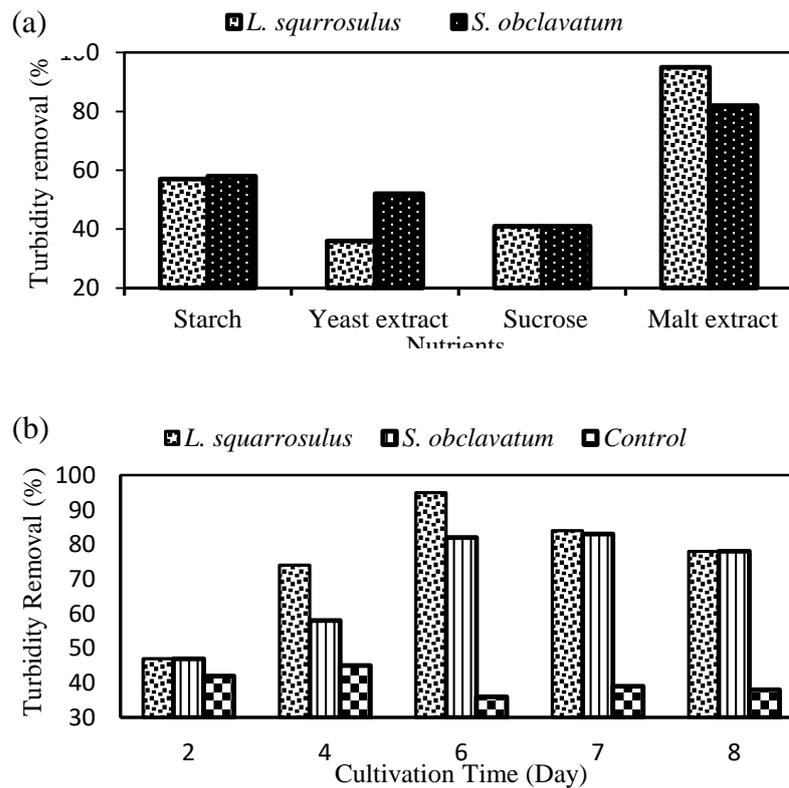


Fig. 1: The effects of nutrient and cultivation time on myco-flocculant production.
a) Screening of potential nutrient, b) Culture time with malt extract.

The highest myco-flocculant production from *L. squarrosulus* and *S. obclavatum* were obtained in malt extract media. Malt extract was used as co-substrate to enhance the separation and filtration process of treated sludge by fungal growth formation [24]. Aljuboori et al. [25], reported that the highest coagulant production by *Aspergillus niger* was observed when the C/N ratio increased up to 20/1 with a flocculating rate of 79% respectively, but that further increase in the C/N ratio slightly decreased the production. Figure 1 (b) shows the cultivation time with flocculation activity in terms of turbidity removal rate of myco-coagulants. The culture during the early stationary phase showed the highest (95% and 82% at 6 days) flocculating rate of *L. squarrosulus* and *S. obclavatum*, respectively. Results showed the flocculating rate decreased slowly after 6 days, suggesting these strains may be secreting deflocculation enzymes. A similar result was reported in the cultivation of *A. parasiticus* [20] and *B. licheniformis* [26]. Consequently, a period of 6 days was chosen as the culture time for the subsequent experiments.

3.1.2 Effect of Media Concentration, Initial pH, and Inoculum Size

Figure 2 shows the effect of malt extract concentration on the flocculating rate of two myco-coagulants. *L. squarrosulus* and *S. obclavatum* increased myco-flocculants at 0.1% (w/v) and 0.25% concentration, respectively. The flocculating activity of two fungal strains was poor without malt extract concentration in the culture (0%). Small growth was found from the two fungal strains without nutrients (malt extract), suggesting that the strains may not be able to secrete any metabolites due to lack of growth.

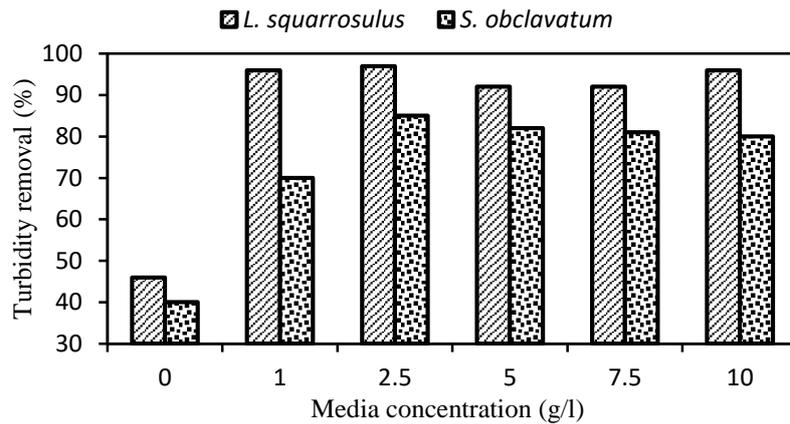


Fig. 2: Effect of media concentration of the flocculation activity on myco-flocculants.

Figure 3 shows the effect of the initial pH of the culture medium on bio-coagulant production. The flocculating activity increased when pH was varied from 5 to 7 with the maximum flocculating activity of 95%. The removal rate (77%) decreased at an alkaline pH (8) for both of the cultures. Salehizadeh et al. [1] investigated that the initial pH of the production medium influences the electric charge of the cell and oxidation-reduction potential, which in turn affects the nutrient absorption and enzymatic reaction.

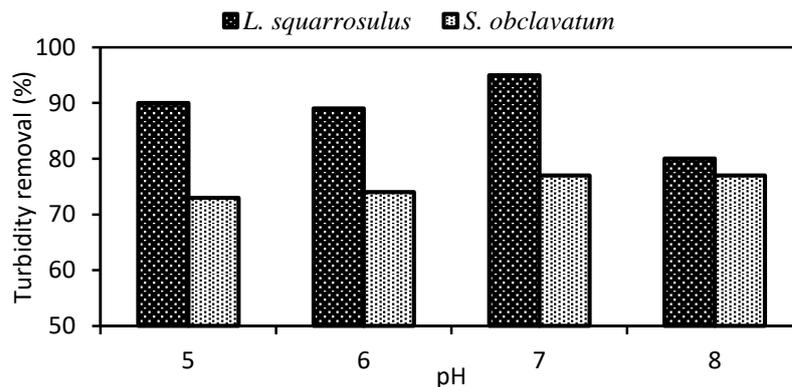


Fig. 3: Effect of the initial pH of the culture medium on the flocculating activity of myco-flocculants.

The flocculating rates obtained from the culture inoculated with 1 to 4% (v/v) inoculum size of two fungal strains are shown in Fig. 4. The use of 2 to 3% of *L. squarrosulus* inoculum dose in production medium recorded the highest flocculating rate of 96%. *S. obclavatum* was gradually decreased when inoculum dose was increased due to excessive overlapping of cells, resulting in inhibition of coagulant production. Aljuboori et al. [13] reported an optimal coagulant production by *Aspergillus flavus* at 2% (v/v) inoculum size, whereas a higher inoculum size of 10% (v/v) was most suitable for coagulant production by *Rhizopus sp.* [21]

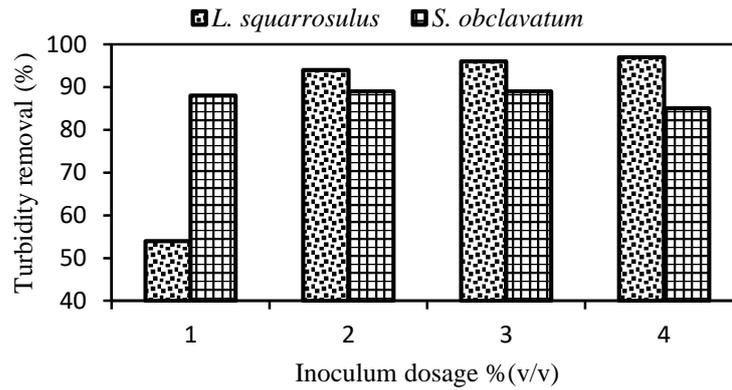


Fig. 4: Effect of inoculum dose of culture medium on myco-flocculants production.

3.1.3 Compatible Mixed Culture

Figure 5(A) shows ten day old single and mixed culture plates of *L. squarrosulus* and *S. obclavatum*. Figure 5(B) shows the turbidity removal (%) of single and mixed cultures. The flocculation rate was higher at 88% of mixed culture compared to the single culture of *S. obclavatum* (78%). The mixed culture showed significant growth of *L. squarrosulus* in the PDA plate, which indicated that the compatible mixed culture of L/S may have produced more substances to enhance flocculation to reduce turbidity compared to single *S. obclavatum* culture. Alam et al. [12] reported the fungal mixed culture of *Aspergillus niger* and *Penicillium corylophilum* was enhanced the separation process of wastewater sludge. Two strains of *Rhizopus sp.* produced a compound coagulant that was used to reduce turbidity of 91.1% from potato starch wastewater [21].

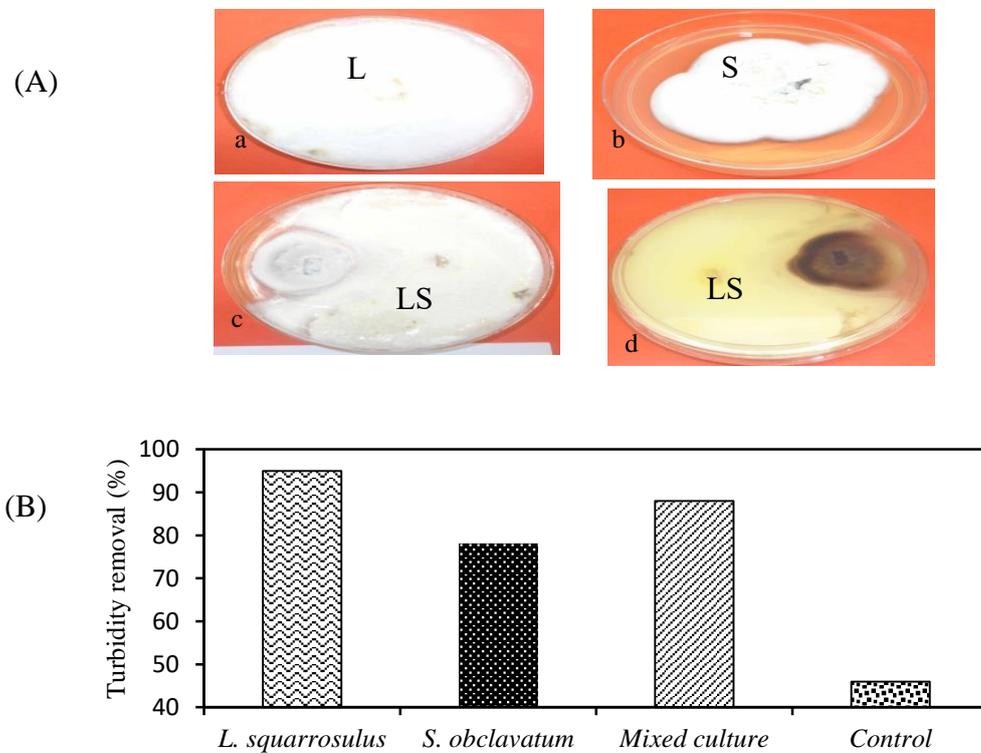


Fig. 5: Compatible mixed culture (A) *L. squarrosulus* (A-a), *S. obclavatum* (A-b), *L. squarrosulus* and *S. obclavatum* (L/S) front side of plate (A-c), L/S reverse side of plate (B) Turbidity removal by single and compatible mixed culture.

3.1.4 Myco-coagulants Stability by Jar Test

Figure 6(a) shows that *L. squarrosulus* was stable at a wide range, from pH 4.0 to 8.0 and more than 90% flocculation was achieved within this range. At pH 7.0, the turbidity removal rate was higher for both myco-coagulants and the flocculation rate decreased slowly as the pH increased further. The mixed culture also showed good stability at pH 7.0. Thus, these myco-coagulants are suitable to be applied at acidic and neutral conditions. This may be due to the fact that myco-flocculants show different electric charges at different pH levels and affect the flocculation ability of the bio-flocculant for kaolin particles [27].

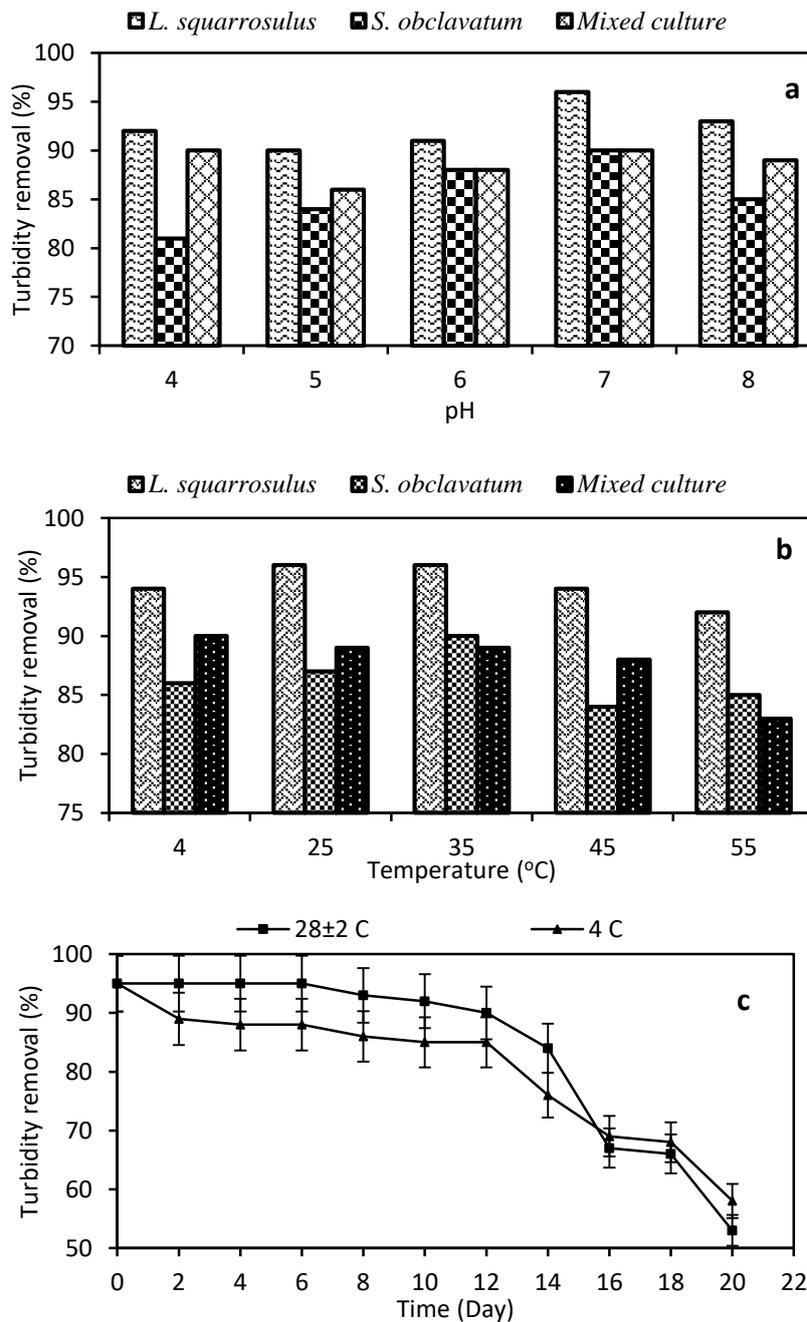


Fig. 6: Myco-flocculant stability: a) The pH stability of *L. squarrosulus*, *S. obclavatum* and mixed culture; b) Temperature stability of *L. squarrosulus*, *S. obclavatum* and mixed culture; c) Time Stability of myco-flocculant (*L. squarrosulus*).

The effect of temperature on the flocculating activity of myco-flocculants was investigated (Fig. 6-b). The results showed that both myco-flocculants are fairly temperature-tolerant and had good flocculation activity at a wide range of temperatures. The flocculation ratio varied from 92% to 96% (*L. squarrosulus*) and 84% to 90% (*S. obclavatum*) in the temperature range of 4 °C to 55 °C, and only begins to decrease slightly with further temperature increase from 45 °C to 55 °C. The mixed myco-flocculant showed good stability at 4°C, then gradually decreased in temperature range to 45 °C -55 °C. The highest turbidity removal rate of 96% was observed at 25 °C and 35 °C for the *L. squarrosulus* myco-coagulant. These myco-flocculants were more stable at moderate temperatures (range 25 °C and 35 °C). Similar findings were observed by Aljuboori et al. [9], on the bioflocculant produced by *Aspergillus flavus* with a residual flocculating activity of about 93% at temperature range from 5 to 45 °C, thus indicating a thermostable bioflocculant. The result was similar to reported for the bioflocculant produced by *Aspergillus parasiticus* and it had moderate heat-stability [20].

Figure 6(c) shows the effect of time stability on turbidity removal rate of *L. squarrosulus* produced myco-flocculant. The highest turbidity removal % was recorded at room temperature (28±2 °C) till 12 days then it showed decreased flocculation activity of the myco-flocculant. This may be due to the fact that the metabolites of the myco-coagulant are deactivated gradually after two weeks of storage. However, turbidity removal rate was 85% at low temperature (4 °C) stored coagulant with 12 days. After two weeks, the flocculation activity was decreased gradually and it reached at about 50% at 20 days. This may have occurred due to the deactivation of metabolites.

4. CONCLUSIONS

This study shows that *L. squarrosulus* and *S. obclavatum* produced myco-flocculants that were effective in the removal of turbidity of a kaolin suspension. The optimal conditions of *L. squarrosulus* myco-flocculant production were malt extract concentration 0.1% (g/l), initial pH 7.0 and inoculum dose 3% (v/v) for 6 day cultivation time which the flocculating activity reached above 95%. The two myco-flocculants' stability indicates that they can work at different pH ranges between 4-8 and can be stored at room temperature (25 °C). Finally, both novel myco-coagulants are expected to be potential replacements of chemical coagulants and widely applied in water treatment and other industries.

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