Optimization of BS4 Enzyme Production with Different Substrate Thickness and Type of Trays

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ABSTRAK

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Penambahan enzim BS4 pada pakan dapat meningkatkan nilai gizi bahan pakan ternak. BS4 dihasilkan oleh *Eupenicillium javanicum* BS4 dalam fermentasi substrat padat bungkil kelapa pada bioreaktor baki. Produksi enzim mungkin dapat ditingkatkan dengan aliran udara yang lebih baik melalui pori-pori pada bagian bawah baki dan ketebalan substrat. Tujuan dari penelitian ini adalah untuk mengoptimasi produksi mananase oleh *E. javanicum* BS4 dengan perlakuan ketebalan substrat dan jenis baki. Penelitian dilakukan dengan rancangan acak faktorial (2x2x3) dengan 6 ulangan. Faktor pertama adalah jenis baki (baki tidak berlubang dan baki berlubang), faktor kedua adalah ketebalan substrat (1,5 dan 3,0 cm), dan faktor ketiga adalah lama inkubasi (5 dan 7 hari). Variabel yang diamati adalah kehilangan bobot kering (KBK), aktivitas mananase dan sakarifikasi, kandungan protein terlarut, aktivitas spesifiknya dan *yield* BS4 terhadap bobot substrat. KBK pada hari ke-7 sekitar 31.43- 36.89 tertinggi ditemukan pada perlakuan baki tidak berlubang dengan ketebalan 3 cm pada hari ke-7. Hasil analisis statistik tidak menunjukkan adanya interaksi ketiga faktor, namun terdapat interaksi dua faktor pada aktivitas mananase dan *yield* pada perlakuan jenis substrat terhadap ketebalan dan terhadap lama fermentasi. Aktivitas mananase tertinggi ditemukan pada jenis baki tidak berlubang ketebalan 3 cm pada hari ke-7. Aktivitas sakarifikasi terhadap bungkil inti sawit ditemukan lebih tinggi pada jenis baki tidak berlubang dan fermentasi 7 hari, sedangkan ketebalan tidak berpengaruh. Nilai *yield* aktivitas mananase dan sakarifikasi tertinggi juga dihasilkan pada baki tidak berlubang ketebalan 3,0 cm pada waktu inkubasi pada hari ke-7. Berdasarkan efisiensi energi dan biaya, dapat disimpulkan bahwa kondisi paling optimum untuk menghasilkan enzim BS4 adalah dengan menggunakan baki tidak berlubang, ketebalan 3,0 cm dan lama fermentasi 7 hari.

Kata Kunci: *Eupenicillium javanicum* BS4, Mananase, Baki Berlubang, Fermentasi Substrat Padat, Ketebalan

ABSTRACT

Haryati T. Sinurat AP, Hamid H, Purwadaria T. 2019. Optimization of BS4 enzyme production with different substrate thickness and type of trays. JITV 24(4): 158-165. DOI: http://dx.doi.org/10.14334/jitv.v24i4.2055

BS4 enzyme that is produced from solid substrate fermentation (SSF) on coconut cake with *Eupenicillium javanicum* BS4 in tray bioreactor has been applied as a feed additive. It increases the nutritional value of animal feedstuff. The BS4 production on SSF may be influenced by the better aeration through the perforated trays or by the thinner substrate. The aim of this research is to optimize the production of BS4 with different substrate thicknesses and types of trays. The trial was carried out using a factorial randomized design (2x2x3) with 6 replicates. The first factor was the type of trays: i.e., non-perforated and perforated tray. The second factor was the thickness of the substrate: i.e., 1.5 and 3.0 cm, while the third factor was the duration of fermentation: i.e. 5, and 7 days. The variables observed were moisture content, dry matter loss (DML), mannanase and saccharification activities, soluble protein content, their specific activities, and yield. Statistical analysis showed no interactions between the three factors, but there were interactions between types of trays and substrate thicknesses, as well as type of trays and incubation times on the mannanase activity and yield of mannanase. The results showed that DML was observed on day 7 were around 31.43- 36.89. The highest mannanase activity was observed on the non-perforated tray with 3 cm thickness on day 7. The saccharification activity towards palm kernel meal was better in the non-perforated tray on day 7 but not influenced by The yield value of mannanase and saccharification activities on a non-perforated tray with 3.0 cm thickness on day 7 was also the highest. Based on energy efficiency and the cost of production, it can be concluded that the optimum condition to produce the BS4 enzyme was observed in the non-perforated tray with 3 cm thickness and fermented for 7 days.

Key Word: *Eupenicillium javanicum* BS4, Mannanase, Perforated tray, Solid Substrate Fermentation, Thickness

INTRODUCTION

The feed is the largest contributor to the cost of livestock production, especially when some of the feed ingredients are imported. Imported feed ingredients are normally expensive which makes farmers difficult to gain profit. Farmers may use the agricultural industry by-products such as coconut meal (CM) and palm kernel meal (PKM) in order to reduce the feed price. PKM a by-product obtained in the production of palm kernel oil has been used as an ingredient for poultry feed. However, the inclusion of PKM in poultry feed is still low due to the high content of crude fiber. Its fibers, such as mannan and cellulose caused low digestibility of nutrients (Ketaren et al. 1999). Fibers contained in the feed can be decomposed by the addition of hydrolytic enzymes thus increasing the nutrient digestibility of feed material (Kuhad et al. 1997).

The use of PKM and CM in the poultry feed is still not optimal, due to high fiber content and low energy and protein content. The crude fiber in the PKM is 11.9 – 15.3% (Sinurat 2012) and the crude fiber in the CM is about 23.5-25.5 % (Jaelani 2007). The fiber content in the PKM and CM mostly composed of hemicellulose and dominated by manan and galactomannan (Mairizal 2013). These components of mannan and galactomannan in the PKM and CM are not digested in poultry and reduces the feed nutritional value (Shimizu et al. 2015). The manan and galactomannan are anti-nutrients which can increase the viscosity of the digesta in the gut producing the reduction of absorption of nutrients in the intestine (Mairizal 2013)).

Hydrolysis of mannan into mannooligosaccharides and mannose can be performed using mannanase enzymes. The mannanase enzyme can be applied to increase the nutritional value of poultry feed by hydrolytic activity thus increasing the solubility of crude fiber in the feed (Sigres & Sutrisno 2015). One of the microorganisms that can produce mannanase enzyme is *Eupennicillium javanicum* BS4. The mold has been selected because it produces high-level mannanase and can be well grown in submerged culture containing coconut cake (Purwadaria et al. 2003). In producing enzyme by fermentation, conditions such as temperature, moisture content, aeration, and substrate particle size are important factors. In the solid substrates fermentation, the production process can be affected by several factors such as substrate thickness and aeration condition. Substrate thickness may affect moldy mycelial growth. The thicker the substrate, the mycelium growth in the inside will be inhibited and decreases the production of mannanase enzyme. Aeration is a factor that plays a role in the fulfillment of oxygen demand and together with the release of CO_2 and heat during the fermentation process. A good aeration process will determine the growth rate of microorganisms and may increase the production of enzymes during the fermentation process (Ab Rashid et al. 2012). The enzyme production study by (Maximilian 2017) using a medium-scale tray bioreactor with a capacity of 96 kg showed that the best condition of mannanase enzyme production was observed on a tray with a thickness of 1 cm for 5 days incubation. The thin substrate may support maximum growth of the mold but the total enzyme produce per batch may less. In order to increase the production of mannanase enzymes, a study was designed by increasing the substrate thickness and tray-type in mid-scale tray bioreactors. The presence of holes in the tray is expected to increase airflow on the lower surface so as to reduce airflow resistance to thick substrates and improve metabolism and mold growth during the fermentation process.

MATERIALS AND METHODS

Materials

The substrate materials used for the fermentation was coconut meal, supplied by local feed mill in Bogor. *E. javanicum* BS4 fungi, a collection of Indonesian Research Institute for Animal Production was used as an inoculum to produce the enzyme. The fungi were first grown on Potato Dextrose Agar (PDA) medium for 5 days at room temperature.

Inoculum and fermentation process

Inoculum was prepared by the addition of sterile saline into freshly grown PDA slants. Submerged fermentation was carried out by inoculated 2.5 mL of spore suspension into 250 ml containing 50 ml of potato sucrose broth. The flask was incubated at a rotary shaker at 120 rpm, 28° C for 5 days. Coconut meal as substrate was steamed for 15 minutes at 121ºC, cooled, mixed with mineral mixtures from Mandels minimal medium (Maximillian 2017) and then mixed with 10% of *E. javanicum* BS4 inoculum prepared in the submerged culture. The substrate was put on two (2) kinds of stainless steel trays (tray with holes and trays without holes at the bottom). The amount of the substrate put on the tray was either thin (1.5 cm) or thick (3.0 cm). The fermentation process was carried out in a stainless steel bioreactor. The temperature in the bioreactor was set at 28 ºC and humidity (Rh) of 85 %.

Sample collection and enzyme extraction

Samples were collected at 5 or 7 days and determined for the moisture content and dry matter

loss (DML). The fermented product was extracted in 10x 0.2 M Na-acetate buffer to obtain the enzyme. The mannanase and saccharification activities and their specific activities of the enzyme were determined. DML was calculated by measuring the reduction of DM weight of the substrate before fermentation towards DM weight of fermented products. The yield of enzyme activities towards kg substrate was determined by multiplying the activity in DM to the DM of the product. The DM loss was already considered.

Mannanase activity assay

Mannanase activity was assayed by mixing 0.5 mL of an appropriate diluted enzyme solution with 0.5 ml of 0.5% locust bean gum in 50 mM acetic buffer (pH 5.8) at 40 °C for 30 min. The reaction was stopped by the addition of 1.5 mL dinitrosalicylic acid (Miller 1959). After 5 minutes of boiling, the amount of reducing sugars was determined spectrophotometrically at 540 nm. Mannose was used as a standard. One unit mannanase was defined as the amount of the enzyme in g DM which liberates 1 µmol mannose per minute under the assay conditions.

Saccharification activity

The saccharification activity of the enzyme was determined by measuring the amount of reducing sugars produced from the decomposition of carbohydrates. In this experiment, palm kernel meal (PKC) was used as a source of carbohydrate. Saccharification activity was calculated as U/g DM, while specific activity saccharification of the enzyme calculated as U/mg protein. One unit of activity is the amount of enzyme in g DM that liberates 1 µmol of glucose per minute under assay condition.

The specific activity of the enzyme.

The specific activity of the enzyme was determined towards soluble protein concentration. Protein was determined by the method of Bradford (1976) and Bovine Serum Albumin (BSA) was used as a standard. The measurement of the protein content was expressed in mg protein per g dry weight and specific activity of the enzyme calculated in U/mg of extracellular proteins.

Statistical analyses

All enzyme activities and yields with six replications were analyzed statistically by analyses of variance (ANOVA) in 2 x 2 x 3 factorial design. Differences between treatments was tested by the least significant difference (LSD) when the ANOVA was significant at $P<0.05$ (Steel & Torrie 1980).

RESULTS AND DISCUSSION

Moisture content in the course of fermentation

The moisture content of the substrate after fermentation decreased as the duration of incubation increased in all treatments (Figure 1). The most stable moisture content is detected on the treatment of non-perforated tray with 1.5 cm thickness which is in the range of 50-60%. In the perforated trays with 1.5 cm and 3.0 cm substrate thickness, the moisture content was lower on the 5th and 7th day of incubation, i.e., in the range of 35-45%, although the initial moisture content was similar for all treatments. The reduction occurred due to the high flow of aeration produced an excess of evaporation. In the common aerobic solid substrate fermentation moisture content is increasing towards the duration of fermentation, since respiration produces water molecules. It is possible that the amount of water evaporation was higher than that of the water formation. The excess of evaporation would not occur if control in the bioreactor was working well. The addition of the holes in the perforated trays caused more evaporation which produced very low moisture content on the 5th and 7th days (Figure 1).

In the solid substrates fermentation, water content is one of the important biological factors for fungi growth (Chang & Webb 2017). Moisture content influenced the growth of mold and production of BS4 enzymes (mannanase and saccharification activities). The mycelia already grew in the beginning, when the moisture content was still 50 to 60%. In the late fermentation (5 to 7 days fermentation) the mycelia including the enzyme should have produced especially in the SSF process with had a lot of substrates. However, the loss of excessive moisture reduces the nutrient solubility in the substrate, thus interfering with the mold metabolism activity (Stark & Firestone 1995) including growth and enzyme production. In the top of that data with the moisture content reaching 35% on the perforated trays indicated the condition of the substrate was close to the minimum moisture content for the metabolic activity. Therefore, the enzyme activities of this treatment were the lowest.

Dry matter losses (DML) in the course of fermentation

DML in all trays type and substrate thickness treatments increased when the duration of fermentation increased from day 5 to day 7 (Figure 2). The highest DML was observed in the non-perforated
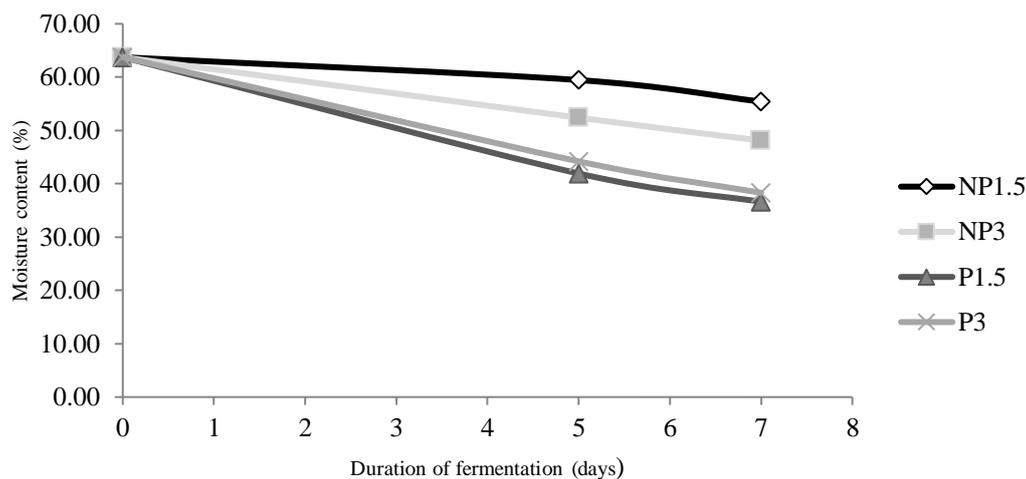
Figure 1. Moisture content of the substrate after fermented on different kind of trays and substrate thickness
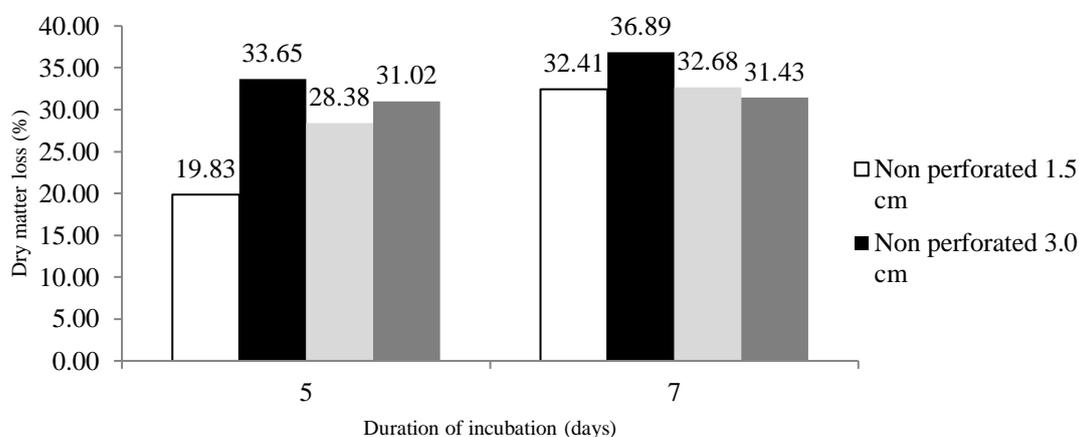*NP: non-perforated; P: perforated

Figure 2. Dry matter loss during the fermentation process

Table 1. Mannanase activity of fermentation product with *E. javanicum* incubated on different tray type and substrate thickness

Type of trays	Substrate thickness (cm)	Mannanase act (U/g DM)
Non perforated	1.5	217.72[a(*)]
	3.0	499.65[c]
Perforated	1.5	346.44[ab]
	3.0	387.11[bc]

(*) Different superscripts indicate statistically significant difference within column P< 0.01

Table 2. Mannanase activity of fermentation product of *E. javanicum* BS4 incubated on different tray type and duration of fermentation

Type of tray	Duration of fermentation (day)	Mannanase (U/g DM)
Non perforated	5	255.34[a(*)]
	7	487.67[b]
Perforated	5	325.90[a]
	7	411.35[ab]

(*) Different superscripts indicate statistically significant difference within the column P< 0.01

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trays with a thickness of 3.0 cm on the 7th day which reached 34% losses. Fermentation in non-perforated trays with 1.5 cm thickness on day 5 showed the lowest (19.83%) DML, but it increased to 32% when the fermentation performed for 7 days. Fermentation in perforated trays with a thickness of 1.5 cm and 3.0 cm shows DML of 31-32% which almost equals those in non-perforated trays with 3.0 cm thickness.

Dry matter losses were increasing during the fermentation process due to the degradation of carbohydrates into CO_2, H_2O, and energy. Therefore, the DML data are always parallel with the growth specificity. The DML values are also related to the water content of the fermented product. In solid substrate fermentation, the moisture content is one of the important biological factors for mold growth (Chang & Webb 2017). In general, the water content of solid substrate fermentation ranges from 30 to 85% (Raimbault 1998). It was already discussed that excessive moisture losses could reduce nutrient solubility in the substrate, thereby disrupting mold growth and reducing DML (Stark & Firestone 1995).

The activity of mannanase produced

There was no significant (P>0.05) interaction between the type of trays, substrate thickness, and duration of fermentation on the activity of mannanase produced. However, there were significant (P<0.05) interactions between type of trays and substrate thickness, as well as the type of trays and incubation time in the mannanase activity. The mannanase activity produced was affected by substrate thickness when the fermentation was performed in the non-perforated trays, while it was not significantly (P>0.05) different when the fermentation was carried out in the perforated trays (Table 1). The highest mannanase activity (499.65 U/g DM) was produced when the fermentation was carried out in the non-perforated trays with 3.0 cm substrate thickness and the lowest (217.72 U/g DM). The highest mannanase activity obtained in the thicker (3 cm) substrate is not in accordance with the hypothesis stated by Ab Rashid et al. (2012) who found that the thinner (0,5 cm) substrate in the trays produced higher mannanase activity than the thicker (1.0 and 1.5 cm) substrate. The inoculum used for the fermentation was *Aspergillus niger* USM F4 and the substrate was palm kernel cake.

The duration of fermentation significantly (P<0.01) affected the mannanase activity produced especially for the non-perforated tray (Table 2). The mannanase activity produced on non-perforated was higher when the fermentation performed for 7 days (487.67 U/g DM) compared to those fermented for 5 days (255.34 U/g DM). When the activity was compared in the perforated tray 7 days incubation was higher 26.2%, even though it was not significantly different (411.35 vs 325.90 U/g DM). Non

significantly data has resulted from the variation that occurred between sample replication. This indicated that the optimum duration of fermentation by using *E. javanicum* is 7 days. This result is in line with DML data, where on non-perforated tray and 7 days fermentation the DML was higher. The optimal duration of fermentation was much influenced by a kind of inoculum and fermentation process. (Sae-Lee 2007) reported that the optimal duration of solid substrate fermentation to produce mannanase was observed at 6 days for *Aspergillus niger*, while there were for 4 and 7 days for *A. oryzae* and *Penicillium* sp. respectively. The same optimal duration was observed with BS4 fermentation and *Penicillium* sp., although the substrate in the manuscript is PKM and the fermentation was carried out in the flask. *Eupenicillium* is very related to *Penicillium*.

Mannanase specific activity, saccharification activity and its specific activities of enzymes produced

The saccharification activities of the enzyme produced are shown in Table 3. Statistical analyses showed that there were no significant (P>0.05) interactions between all factors (the type of tray, substrate thickness, and duration of fermentation) in this experiment. The main factors such as type of trays and duration of fermentation but not the substrate thickness significantly (P<0.05) affect the saccharification activity of the enzyme produced. Fermentation in non-perforated trays produced significantly (P<0.05) higher saccharification activities (85.85 U/g DM) compared to fermentation in perforated trays (56.74 U / g DM). However, the specific mannanase activity (the mannanase activity per mg protein) and the specific saccharification activity (the saccharification activity per mg protein) of enzyme produced in both types of trays were not significantly different (P>0.05). Duration of fermentation also significantly (P<0.05) affect the saccharification activities but not the specific mannanase and specific saccharification activities. Fermentation for 7 days produced significantly (P<0.05) higher saccharification activities (85.45 U/g DM), compared to fermentation for 5 days (57.14 U/g DM).

Saccharification activity shows the activity of enzymes that can break down carbohydrate complex or fibers components in palm kernel cake (PKC) becoming more simple carbohydrates. The higher the saccharification activity indicates that the enzyme has a higher possibility to be applied in animal feed containing high fiber feed ingredients such as PKC. The saccharification activity measured in this experiment was not only due to the presence of mannanase enzymes but also by the presence of other enzymes such as cellulase and xylanase. Besides mannan, PKC also contains other complex

Table 3. Saccharification, specific mannanase, specific saccharification activities of fermentation products of BS4 with tray type, substrate thickness, and duration of fermentation

Treatment		Saccharification Activity (U/g DM)	Specific Mannanase Activity (U/mg)	Specific Saccharification Activity (U/mg)
Type of trays	Non perforated	85.85[b(*)]	8.28	2.48
	Perforated	56.74[a]	10.54	1.81
Substrate thickness	1.5 cm	69.67	9.07	2.65
	3.0 cm	72.65	9.69	1.72
Duration of fermentation	5 days	57.14[a]	8.21	1.77
	7 days	85.45[b]	10.61	2.51

(*) Different superscripts in same column indicate a statistically significant difference between a column in the treatment compared $P<0.05$.

Table 4. The yield of mannanase activity as affected by the type of trays and substrate thickness

Type of trays	Thickness (cm)	Mannanase (U/kg)
Non-perforated	1.5	155, 239[a]
	3.0	320, 917[b]
Perforated	1.5	245, 490[ab]
	3.0	266, 265[b]

(*) Different superscripts indicate statistically significant difference within column $P< 0.01$

Table 5. The yield of mannanase activity as affected by the type of trays and duration of fermentation

Type of trays	Duration of fermentation (days)	Mannanase (U/kg)*
Non-perforated	5	176, 067[a]
	7	315, 151[b]
Perforated	5	233, 956[ab]
	7	279, 687[ab]

(*) Different superscripts indicate statistically significant difference within column $P< 0.01$

Tabel 6. The yield of saccharification activity of the enzyme produced in a different type of trays, substrate thickness, and duration of fermentation

Treatments	Saccharification yield (U/kg substrate)
Type of trays:	
Non perforated	57, 655[b]
Perforated	39, 841[a]
Substrate thickness:	
1.5 cm	49, 993
3.0 cm	47, 711
Duration of fermentation:	
5 days	41, 368[a]
7 days	56, 129[b]

(*) Different superscripts indicate a statistically significant difference between treatment compared $P< 0.05$

carbohydrates such as cellulose and xylan (Abd-Aziz et al. 2009).

It is very possible that the specific activity of mannanase and saccharification were not significantly different, although the activity in the dry matter was different. Those data occurred due to the increase and the decrease of activity are parallel to the protein concentration. The one not expected is the specific saccharification activity, since it implies more enzymes than that mannanase. It seems that the protein concentration also parallels with each enzyme activity. This conclusion might be confirmed if the purification of every enzyme was carried out.

Yield value of mannanase and saccharification activities towards substrate.

The yield value is calculated to evaluate the efficiency in producing enzyme activities including both mannanase and saccharification on a similar amount of substrate in order to decide the most optimum method of producing the enzyme. The evaluation already considered DML. Statistical analysis on yield of mannanase activity showed that there were interaction between types of trays and substrate thickness, as well as type of trays and duration of fermentation, while that of saccharification activity showed that there was no interaction between all factors (type of trays, substrate thickness, and duration of fermentation) in this experiment (Table 4, 5, and 6). Following data of mannanase activity, the highest yield of mannanase activity was observed in the non-perforated tray at 3 cm substrate thickness and 7 days duration of fermentation. The treatment shows the most efficient enzyme production, although it also resulted in the highest DML. The high activity covered the loss of the substrate.

The saccharification yield was significantly affected by the main factor effect of type of trays ($P<0.01$) and the duration of fermentation ($P<005$), but not significantly ($P>0.05$) affected by substrate thickness. The enzyme produced in non-perforated trays yields higher saccharification activity compared with those produced in perforated trays (57.655 U/kg vs 39.841 U/kg substrate). Fermentation carried out for 7 days produced an enzyme with significantly ($P<0.05$) higher saccharification yield (56.129 U/kg substrate) than that produced for 5 days (41.368 U/kg substrate). The yield of saccharification activity from 3 cm thickness was similar to the 1.5 cm, therefore on the base of BS4 enzyme activities and yield toward substrate it can be concluded that BS4 production was best carried out in the non-perforated tray with 7 days duration of fermentation and 3 cm thickness.

In the fermentation process, the value of $Y_{p/s}$ or yield of a product towards substrate is generally

calculated in the same unit, as occurs in ethanol fermentation with a yield of 0.44 g/g (Mathew et al. 2015). The value is always below 1, especially if both compounds are built from similar molecules like glucose as the substrate and ethanol as the product. Both molecules contain carbon, oxygen, and hydrogen. Although the $Y_{p/s}$ is lower than 1, the process is economic. In this study, efficiency is evaluated based on the comparison of enzyme activity against the substrate (Unit/kg substrate). Enzyme activity was measured as the product since it works as a biocatalyst. The yield value was much higher than 1, however, it does not imply economic benefit. The economic value should consider all the costs of the production and the effectivity of the enzyme in the application. The yield data in the experiment only show the efficiency of the substrate for each treatment.

CONCLUSION

The type of trays and substrate thickness affects the growth of *E. javanicum* BS4, thus affecting the production of enzymes in medium-scale tray bioreactors. The use of perforated trays may lead to excessive oxygen transfer on the substrate so that the moisture content becomes very low and dry. The thicker substrate was better to maintain the water content during the fermentation process than the thinner substrate. Based on mannanase and saccharification activities as well as the yield of activities towards the substrate, BS4 production was best produced on a non-perforated tray with 3.0 cm thickness for 7 days fermentation.

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