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Short Communication

Isolation, screening and identification of mannanase producer microorganisms

(Pemencilan, penyaringan dan pengenalpastian mikroorganisma penghasil enzim mannanase)

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Keywords: palm kernel cake, mannan, mannose, glucosamine, animal feed, *Aspergillus niger*

Abstract

Palm kernel cake (PKC) is used widely in the animal feed industry but has limited use in poultry feed due to its high fibre and low protein contents. The major component of the fibre is mannan which is insoluble and difficult to digest. Therefore, isolation of local potential microbes capable of degrading mannan was explored from various sources such as peat soil, rotten oil palm trunks and raw PKC. The isolates were screened based on the clearing zone method on selective agar media containing Azo-carob-galactomannan as substrate. A total of 36 isolates were screened and only 13 had a clear zone ranging from 3.42 ± 0.02 mm to 5.44 ± 0.06 mm which were used for further analysis. These isolates were incubated in shake flasks at 35 °C for 48 h with 10 g/litre PKC as substrate. The best enzymes producer was isolate IBRL F16.A4 with specific mannanase enzyme acitivity of 17.82 ± 0.05 U/mg, production of glucosamine at 9.84 ± 0.11 mg/g and mannose at 9.54 ± 0.06 mg/g. The isolate IBRL F16.A4 was identified as *Aspergillus niger* IBRL F16.A4 using a scanning electron microscope.

Introduction

The largest single cost item in the poultry industry is feed. The high price of imported feed ingredients such as maize and soybean has resulted in high costs of poultry production. In 2011, Malaysia spent US$906 million in total feed ingredient imports and this value was 10.4% more than the amount recorded in 2010 (US$820 million). The poultry industry in Malaysia can be considered as modern, highly competitive and successful, but the industry totally depends on imported feedstuffs (Wan and Hong 2009).

As a result the country is less competitive in the poultry world market. The importation of large quantities of grain for stock feeding has resulted in great loss of foreign exchange and this has become a major concern to the poultry industries. Attempts to produce home-grown feed crops, particularly maize and soybean, have not been successful. Under the current situation, the production of feed grain is technically feasible but not an economically attractive venture. For these reasons, other feed resources have to be considered. One of the examples is palm kernel cake

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(PKC). Production of PKC in Malaysia is in abundance throughout the year and has increased more than 43% since 1999 (1.62 to 2.39 million metric tonnes in 2011) (Mohammad et al. 2012). This justified its utilization as feed for poultry, thus enabling to save the cost of imported feed (Loh 2002). Palm kernel cake can be considered as a reliable supply of raw materials compared to other by products. The other advantage of PKC is its low cost compared to soya bean meal. Price comparison between soybean cake and PKC showed that soybean cake costs US$550/MT while PKC costs US$150/MT (Ayob et al. 2011). PKC can be used to substitute at least 20% of the imported feed which is mainly corn (Mohammad Amizi et al. 2012).

However, the major components of PKC are mannan and cellulose to a lesser extent (Jaafar and Jarvis 1992). The mannan of PKC is hard and crystalline, with a degree of low substitution of galactosyl residue compared to the seed of galactomannans. As a result, the mannan is insoluble and resistant to enzymatic degradation. Due to its fibrous nature, its use in monogastric diets is not favourable. To digest the fibre, enzymes produce by microorganims can be added. The removal of the fibre will liberate the starch and protein which masks the cell structure and this can lead to increase in the metabolisable energy and protein utilization by the poultry.

A saccharification process has to be carried out to hydrolyse the fibre (polysaccharide) to simple sugar (mannose) so that the poultry can digest it (Hagglund et al. 2003). Microorganisms grown on the PKC can be isolated and tested for its ability to produce the enzyme mannanase. Many researchers have studied the bioconversion of mannan to simple sugars using copra mannan, guar galactomannan and carob galactomannan (Ademark et al. 1998; Tse and Chinshuh 2004; Sumitra et al. 2005; Sabu et al. 2005; Illuyemi et al. 2006). The use of enzymes in the poultry industry has become a common practice in improving the nutritive value of their diets. Numerous publications have indicated the importance of enzymes, particularly when certain indigestible and viscous compounds are present in the diets. This condition coupled with advances in microbial technology, has improved our understanding of enzymes and their target substrates. Great success has been achieved in using enzymes such as *β-glucanase, xylanase* and *β-mannanase* on these feedstuffs (Jackson et al. 2004).

The main nutritional problem of PKC, either physically or physiologically is the content of the indigestible fibre. Recent findings have shown that problems related to high indigestion of fibrous materials can be overcome by enzymatic treatment which has been performed with many other feedstuffs (Kim et al. 2003). The use of these enzymes will help to accelerate hydrolytic processes of isolated mannan-based polysaccharides and cellulose (Balasubramaniam 1976) and may improve the digestion of PKC which is used as poultry feed. Therefore, the objective of this study was to identify the locally available microbes that are capable of degrading mannan.

Materials and methods

Chemicals and substrates

Bacteriological peptone and yeast extract were obtained from Oxoid Laboratories. Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) and calcium phosphate crystal (KH_2PO_4) were obtained from Fulka Biochemica. Standard for determination of glucosamine standard curve was obtained from Sigma G-4875 (Glucosamine-HCl, 99%) whereas for protein standard curve, Bovine Serum Albumin (BSA) was obtained from Sigma Chemicals. All other chemicals used in this study were of reagent and analytical grades from Sigma Chemicals. Potato Dextrose Agar (PDA) was obtained from Oxoid Limited, Basingtoke, Hamsphire, England.

The substrate, palm kernel cake (PKC) was collected from Kilang Isi Sawit, Felda Serting in Jempol, Negeri Sembilan.

Meanwhile, a commercial mannan, azo-carob-galactomannan and locust bean galactomannan were obtained from Megazyme International Ireland Limited, Co. Wicklow, Ireland.

Source of microorganisms

Samples from peat soils, rotten oil palm trunks and raw palm kernel cake (PKC) were collected randomly from United Plantation (UP), Hutan Melintang, Perak and were used as sources of microorganisms for screening, isolation and identification.

Isolation of mannanase producing fungi

Qualitative screening The isolation of fungi was carried out on Potato Dextrose Agar (PDA) using the sample obtained from peat soils, rotten oil palm trunks and raw PKC. A tenfold dilution was made by mixing 1.0 g of the sample in 10 ml sterile distilled water and shaken vigorously for 10 – 15 min using a vortex (Khampheng et al. 2006). Then, 1.0 ml of the mixture was transferred on the surface of PDA plates and spread plate was performed using an L-shaped glass rod. The inoculated plates were incubated at 37 ± 1°C for 5 – 7 days. The fungal isolates formed were subcultured to purity and examined for mannanase activity.

Screening for mannanase activity was performed on a modified carbon limited basal growth medium (Kusakabe and Takahashi 1986) which contained (g/l): peptone 9.0; yeast extract, 1.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; technical agar, 15.0 and a carbon source of commercial mannan, azo-carob-galactomannan, 2.0. The pH of the medium was adjusted to 5.5. Positive mannanase producer isolates were detected based on the clear zone formed on the medium around the colony of the isolates after 24 h incubation.

Quantitative screening The isolates that produced clear hydrolysis zones were then purified and sub-cultured on fresh PDA slants, and stored at 4 ºC prior to use. These isolates were then grown in a liquid medium consisting of (g/l): peptone, 9.0; yeast extract, 1.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; and PKC, 10 as a carbon source (Kusakabe and Takahashi 1986). The fungal spores were added into 150 ml of growth medium in an Erlenmeyer flask and kept in an incubator shaker (LSI-1005R, LabTech, Korea) with an agitation rate of 150 rpm and incubated at 35 ± 2 °C for 48 h. The quantity of mannanase activity and other parameters including protein concentration (mg/g), glucosamine (mg/g), mannose (mg/g) and final pH were measured after 48 h of cultivation period. All the experiments were carried out in triplicates.

Identification of isolates

Fungal identification was carried out based on the colony morphology and structural characteristics as observed under a light (Olympus BHB, Japan) and scanning electron microscope (Leica Cambridge S300). The fungal characteristics were described and identified based on both macroscopic characters (colony growth, colony surface) and microscopic characters (aseptate hyphae, branched hyphae, conidiophore, vesicle) using the description given by Harley et al. (1995), Hoog et al. (2000) and Samson et al. (2010).

Analyses of endo-1,4-β-mannanase activity, protein concentration, reducing sugar concentration and fungal growth

The *endo-1,4-β-mannanase* activity was determined by the method of McCleary (1988), with azo-carob-galactomanan (Megazyme, Ireland) as the substrate. About 0.5 ml of isolate was added into 0.5 ml of substrate (Azo, 2%; w/v). The activity was determined based on the standard curve of *endo-1,4-β-mannanase* activity. The blank was prepared by adding ethanol to the substrate before the addition of the enzyme. One unit of *endo-1,4-β-mannanase* was defined as the amount of enzyme required to release 1 μmole of mannose reducing sugar

equivalents per min under the defined assay conditions.

Protein assay was determined using the Bio-Rad Protein Assay Kit based on the method of Bradford (1976). The concentration was determined based on the reference protein standard curve using bovine serum.

Reducing sugar concentration was determined using the dinitrosalicyclic acid (DNS method) as described by Miller (1959). Standard mannose (reducing sugar) at a concentration in the range of 0.25 – 8.00 mg/g was prepared. The concentration of the reducing sugar was determined based on a standard curve.

The growth of the fungus was determined based on the total glucosamine content. Total glucosamine was determined after acid hydrolysis (Kremnicky et al. 1996). The glucosamine concentration was determined based on a standard curve (Jacyno and Thrall 2004).

Results and disscussion

Selection of potential isolates for mannanase production

A total of 36 isolates were screened and capable of growing on solid medium containing commercial mannan, azo-carob-galactomannan as a carbon source with a diameter of the clear hydrolysis zones ranging from 0.42 – 5.44 mm (*Table 1*). Out of these 36 isolates, 13 exhibited diameters of clear hydrolysis zones ranging from 3.42 ± 0.02 – 5.44 ± 0.06 mm.

Table 2 shows the qualitative screening of the 13 isolates in a liquid growth medium after 48 h of incubation. It was found that the 13 isolates produced specific mannanase activity in the range of 4.37 ± 0.04 – 17.82 ± 0.05 U/mg, with production of glucosamine and mannose in the range of 8.08 ± 0.06 – 9.98 ± 0.11 mg/g and 7.23 ± 0.06 – 9.64 ± 0.05 mg/g respectively.

Although there are large variations in the enzyme productivity based on growth performance and mannanase activity, isolate IBRL F16.A4 showed the highest specific

Table 1. Isolation and quantitative screening of mannanase producing isolates on [1]modified carbon limited basal growth medium

No.	Strain code	[2]Diameter measurement (mm)
1	F16.A1	1.13 ± 0.01
2	F15.B1	1.23 ± 0.12
3	F15.A1	2.18 ± 0.11
4	Y16.B1	0.42 ± 0.06
5	F16.A2	1.97 ± 0.03
6	F15 B2	1.31 ± 0.03
7	Y16.C1	0.47 ± 0.04
8	F15.B3	1.32 ± 0.13
9	F16.B1	1.09 ± 0.11
10	F15.C1	2.18 ± 0.08
11	F15.B4	2.13 ± 0.03
12	Y15.B1	2.97 ± 0.02
13	F15.C2	2.10 ± 0.12
14	Y15.B2	2.24 ± 0.13
15	F15.B5	3.32 ± 0.04
16	F16.A3	4.08 ± 0.11
17	F16.A4	4.32 ± 0.08
18	F15.B6	1.43 ± 0.02
19	F16.A5	4.76 ± 0.02
20	F15.A2	3.98 ± 0.02
21	F16.B2	3.14 ± 0.04
22	F15.A3	5.44 ± 0.06
23	F16.C1	3.21 ± 0.03
24	F15.A4	4.32 ± 0.05
25	F16.B3	2.08 ± 0.04
26	F15.A5	3.42 ± 0.02
27	F16.C2	2.21 ± 0.07
28	F15.A6	3.21 ± 0.04
29	F16.A6	4.51 ± 0.09
30	F15.B7	2.24 ± 0.05
31	F16.A7	4.32 ± 0.08
32	F15.C3	3.48 ± 0.09
33	F16.A8	3.72 ± 0.1
34	F15.C4	3.89 ± 0.1
35	Y15.A1	1.32 ± 0.14
36	F15.B8	3.47 ± 0.06

[1]Modified carbon limited basal growth medium composition (g/l): peptone 1.0; yeast extract, 0.5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; technical agar, 15.0, azo-carob-galactomannan, 2.0 and pH 5.5 for 48 h incubation at 35 ± 1 °C.
[2]Each value represents the mean of 3 replicates experiments ± standard deviation.
Coding system: F15.A2 stands for a fungus isolated from palm kernel cake (in second serial number), Y = actinomycetes, F = fungi, A = raw palm kernel cake (PKC), B = peat soil and C = rotten oil palm trunks

Table 2. Qualitative screening of 13 isolates in liquid medium in a shake flask system

Strain code	[2]Specific mannanase activity (U/mg substrate)	[2]Glucosamine (mg/g substrate)	[2]Mannose (mg/g substrate)	Final pH
F16.A3	13.39 ± 0.01	9.98 ± 0.01	7.93 ± 0.11	4.3
F16.A4	17.82 ± 0.05	9.84 ± 0.11	9.54 ± 0.06	4.5
F16.A5	13.32 ± 0.06	9.18 ± 0.04	9.27 ± 0.08	4.8
F15.A2	13.87 ± 0.03	8.28 ± 0.04	7.89 ± 0.11	4.7
F15.A3	13.31 ± 0.11	8.98 ± 0.03	7.23 ± 0.06	4.6
F15.A4	7.02 ± 0.03	9.44 ± 0.07	9.64 ± 0.05	4.5
F15.A5	13.32 ± 0.11	9.78 ± 0.06	9.43 ± 0.08	4.7
F16.A6	13.61 ± 0.06	9.98 ± 0.11	8.43 ± 0.13	4.6
F16.A7	8.42 ± 0.03	9.32 ± 0.12	9.04 ± 0.06	4.6
F16.A8	13.82 ± 0.08	9.21 ± 0.04	9.07 ± 0.09	4.7
F15.C3	9.31 ± 0.01	8.08 ± 0.06	8.91 ± 0.03	4.2
F15.C4	13.19 ± 0.10	9.04 ± 0.07	9.14 ± 0.08	4.4
F15.B8	4.37 ± 0.04	9.08 ± 0.04	9.23 ± 0.06	4.5

[1]Liquid medium composition (g/l): peptone, 9.0; yeast extract, 1.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; and palm kernel cake, 10. Culture conditions: pH 7.0; 150 rpm; 35 ± 2 °C for 48 h incubation.
[2]Each value represents the mean of three replicates

mannnase activity of about 17.82 ± 0.05 U/mg, and 9.84 ± 0.11 mg/g and 9.54 ± 0.06 mg/g of glucosamine and mannose concentrations respectively. This isolate was selected as the best mannanase producer and was chosen to be used for subsequent experiments. The final pH of cultivation medium was 4.5 which was obtained after 48 h of cultivation at 35 ± 2 °C.

Identification of the isolate IBRL F16.A4

Isolate IBRL F16.A4 was a fungus which was isolated from raw PKC. The pure colony of the fungus was subcultured on PDA slants and incubated at 35 ± 2 ºC. The initial colour of the colony was white and then became black after maturation of conidia. After 7 days of incubation, the reverse side of the growth plate turned from pale yellow to brown and the colony produced radial fissures in the agar plate (Hoog et al. 2000; Jesenka et al. 1992). The IBRL F16.A4 colonies were black in colour on the agar surface due to sporulation, with white to yellow on the reverse side of the agar medium.

Observation under a light microscope (*Figure 1*) showed that the fungus produced black colour spores (*Figure 1a*) with hyaline branched hyphae (*Figure 1b*) and septate hyphae (*Figure 1c*), and also rounded vesicle heads (*Figure 1d*) that radiate initially and splitting into columns at maturity. The fungus was biseriate (vesicles produced sterile cells known as metulae that support the conidiogenous phialides). Conidiophores were long smooth and hyaline, becoming darker at the apex and terminating in a globose shape. Metulae and phialides covered the entire vesicle.

Figure 2 shows the scanning electron microscope (SEM) micrographs of the isolate IBRL F16.A4 structures. It was shown that the fungal structures consisted of smooth and colourless conidiophores (*Figure 2a*). The vesicles (*Figure 2b*) were globose in shape and were held at the tip of the conidiophores. The vesicles were covered by metulae, which carried the phialides and chains of conidia. The conidia were spherical, affluent and black in colour (*Figure 2c*). A closer view of the IBRL F16.A4 conidia revealed a black, rough surface and globose shape ranging in size between 3.50 and 3.84 μm (*Figure 2d*). These characteristics were similar to those described by Harley et al. (1995) in their observation of the fungus, *Aspergillus*

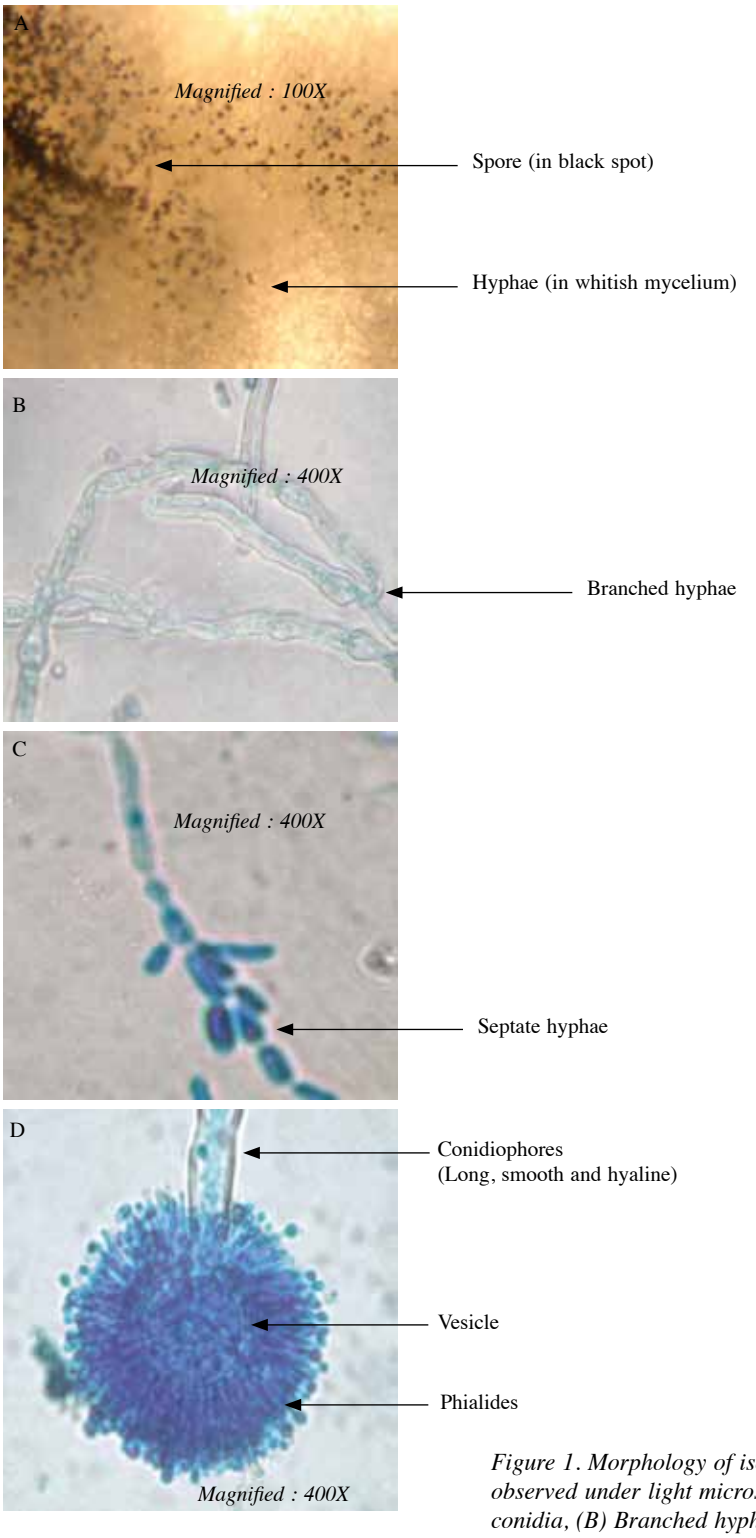

Figure 1. Morphology of isolate IBRL F16.A4 observed under light microscope. (A) Black spores/ conidia, (B) Branched hyphae, (C) Septate hyphae and (D) Typical Aspergillus head

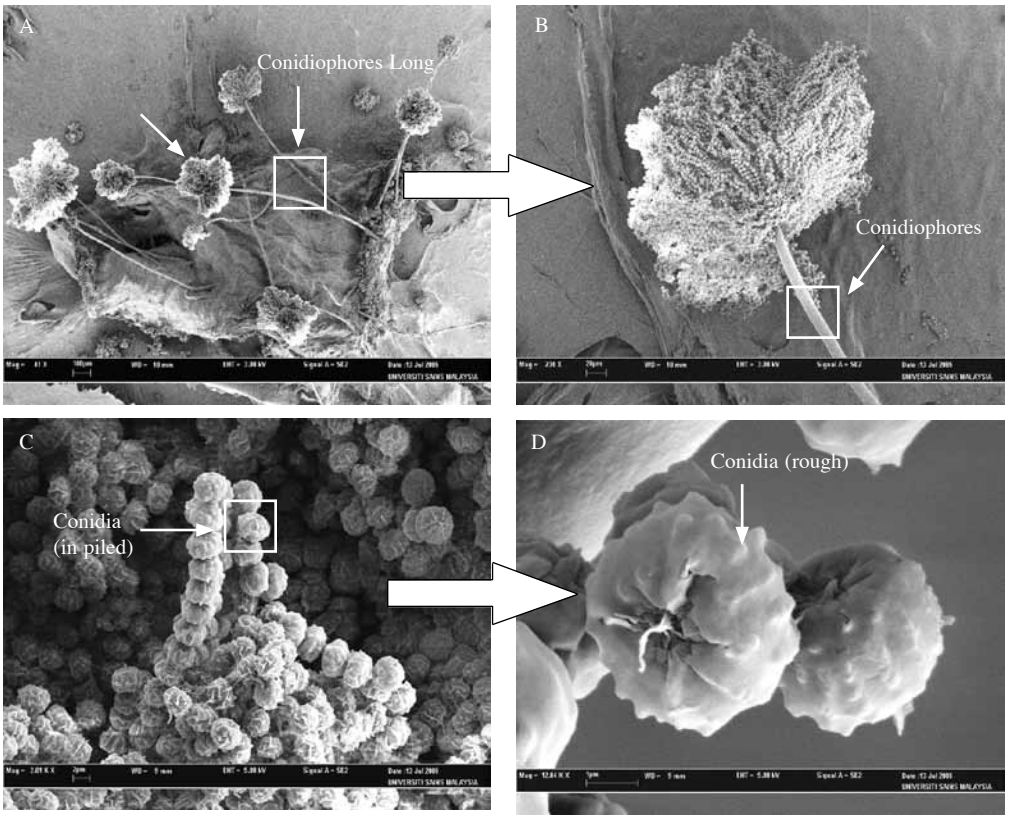

Figure 2. SEM micrographs of isolate IBRL F16.A4 after 7 days of incubation. (A) Morphology of the fungal structure, (B) the vesicle, (C) chain of conidia and (D) black rough surface and globose shape

sp. Snell and Schweiger (1949) also gave a similar description but observed that the mycelial morphologies of acidogenic *Aspergillus niger* as short, swollen filaments with swollen tips. These similarities can be used to confirm the identity of isolate IBRL F16.A4 as *Aspergillus niger*.

Conclusion

Thirty six isolates capable of hydrolysing an azo-carob-galactomannan as a carbon source on solid agar medium were isolated from samples of peat soils, rotten oil palm trunks and raw PKC. Thirteen of the isolates that produced a diameter of clear zone ranging between 3.42 ± 0.02 and 5.44 ± 0.06 mm were selected for mannanase production in a liquid cultivation medium. The best enzyme producer was isolate IBRL F16.A4 with a specific mannanase acitivity at 17.82 ± 0.05 U/mg, production of glucosamine at 9.84 ± 0.11 mg/g and mannose at 9.54 ± 0.06 mg/g after 48 h of incubation. The mannan degrading enzyme producer was identified microscopically as *Aspergillus niger* IBRL F16.A4 by observation using a scanning electron microscope.

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Abstrak

Hampas isirung kelapa sawit (HIKS) digunakan secara meluas dalam industri makanan ternakan tetapi penggunaannya sebagai makanan poltri agak terbatas kerana kandungan serat yang tinggi dan protein yang rendah. Komponen utama dalam serat ini ialah mannan yang tidak mudah larut dan dihadam. Justeru, organisma mikrob pencilan tempatan yang mampu menghurai mannan dikaji daripada pelbagai sumber seperti tanah gambut, batang kelapa sawit yang reput dan HIKS mentah. Pencilan ini disaring berdasarkan zon penjernihan pada medium agar yang bersifat pemilih mengandungi azo-carob-galactomannan sebagai substrat. Sejumlah 36 pencilan disaring dan hanya 13 dipilih untuk analisis seterusnya berdasarkan penghasilan zon penjernihan dalam lingkungan 3.42 ± 0.02 hingga 5.44 ± 0.06 mm. Mikrob pencilan ini dieram pada suhu 35 °C selama 48 jam di dalam kelalang goncangan yang mengandungi 10 g/liter HIKS sebagai substrak. Penghasil enzim yang terbaik ialah pencilan IBRL F16. A4 dengan aktiviti enzim mannanase spesifik 17.82 ± 0.05 U/mg, penghasilan glukosamina 9.84 ± 0.11 mg/g dan mannose 9.54 ± 0.06 mg/g. Pencilan IBRL F16.A4 dikenal pasti sebagai *Aspergillus niger* IBRL F16.A4 melalui pemerhatian yang dibuat menggunakan mikroskop pengimbas elektron.

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