Survey of coastal mangrove fungi for xylanase production and optimized culture and assay conditions

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Abstract: Xylanase activity was detected among 34 of 77 fungal isolates derived from decaying wood, debris and soil samples collected in coastal mangrove environment of Hong Kong. Of those, three isolates CY2809 (*Staganospora* sp.), CY4786 and CY5040 were chosen for comparison of xylanase production in parallel to HU5048 (*Aspergillus awamori*), a terrestrial, highly productive isolate. Based on the assessment of mycelial biomass, xylanase activity and content of xylose-equivalent reducing sugars in their liquid cultures, the isolate CY4786 was best for xylanase production in a basal medium containing birchwood xylan (10.0 g/L) as a sole carbon source, yeast extract (2.5 g/L) and sea salts (15.0 g/L) with initial pH 7.8. When assayed at the optimized regime of 50℃ and pH 4.6, the activity of xylanase produced by CY4786 in 7d liquid culture at 25℃ reached 1.07×10^4 unit/mL. The results indicate that the mangrove fungi act as hemicellulose decomposers in the mangrove environment where highly xylanase-productive isolates can be searched for exploitation. A discussion is given on the possible use of the content of xylose-equivalent reducing sugars as an index to simplify conventional xylanase activity assay method for fungal isolate survey.

Key words: Mangrove Fungi, Xylanase, Reducing Sugar, Basal Medium, Optimized Culture Condition

The breakdown of xylan, a major component of hemicelluloses, was confirmed to be associated with the activity of microbial xylanase[1~4]. Mangrove fungi play an important part in the decomposition of ligno-cellulose materials in tropical or subtropical coastal ecosystems where mangrove debris is abundant. Although a diversity of fungi is associated with the decomposition, the involved processes have not been well understood. Previous studies on wood decaying activities in the coastal ecosystems were primarily restricted to *in situ* examination of rotting symptoms and measurement of wood weight loss. The activities of ligno-cellulolytic enzymes such as cellulases, laccases and peroxidases in the mangrove fungi have been reported[5,6]. However, little attention has been given to xylanase, a major enzyme very likely to be involved in the breakdown of xylan that constitutes hemicelluloses in mangrove wood. Moreover, xylanase has been widely used as an animal feed additive[7~9] and is also of great potential for use in paper and pulp industries[10,11]. The xylan-hydrolyzed products may also be converted to liquid fuel, single cell proteins, solvents and low-calorie sweeteners[12,13]. Thus, investigating the potential of the mangrove fungi in xylanase production is not only important for a better understanding of their role in the wood decaying processes in the high-salinity coastal ecosystems but also helpful for identifying high yielding xylanase isolates.

In the present study, up to 77 isolates of mangrove fungi were examined for *in vitro* xylanase production. Selected isolates were further evaluated for their capability to produce xylanase under different culture and assay conditions. Optimal conditions including salinity, temperature, nitrogen sources, pH and incubation time were determined for the production of xylanase by the best isolate selected.

1 Materials and Methods

1.1 Source of fungal isolates

Samples of decaying wood, leaf debris and soil beneath the debris or wood were collected from the mangroves of Sai Keng in Three Fathoms Cove, Hong Kong. To isolate fungi from the samples, small pieces of mangroves materials (~5mm in diameter) were immersed in 0.5% sodium hypochlorite for 3s and rinsed with distilled water. After removal of excessive water with filter paper, the small pieces were individually placed onto plates of Oxoid (Hampshire, England) corn meal agar prepared with Sigma (St. Louis, MO, USA) sea salts 15g/L (CMASS), supplemented with

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0.25g/L penicillin G (1610 unit/mg) and 0.25g/L streptomycin sulfate (761unit/mg) to avoid bacterial contamination. One gram of the soil sample was suspended in sterile Ringer's solution and diluted stepwise to a concentration of 10^{-2}, 10^{-3} and 10^{-4}. Subsequently, every 0.2mL of each diluted soil suspension was plated onto a CMASS plate. After incubation at 25℃ for 2 ~ 3 d, fast-growing fungal colonies were separately transferred to fresh CMASS plates for further purification. A total of 56 isolates were obtained from the samples. Another 21 isolates of mangrove fungi obtained from the Culture Collection of the Department of Biology and Chemistry, City University of Hong Kong were also included in the following first-run assays. A terrestrial, highly xylanase-productive isolate, HU5048 (*Aspergillus awamori*), from the collection of Zhejiang University was used as a positive control.

1.2 Screening of fungal isolates

The components of the medium used in the first-run assays included Sigma birchwood xylan 10.0g/L, Difco (Detroit, USA) yeast extract 0.5g/L, Oxoid tryptone 0.5g/L, Sigma sea salts 15.0g/L and Oxoid agar # 3 15.0g/L. After the medium were autoclaved at 121℃ for 15min and cooled to ~ 50℃, 15.0mL of the medium was dispensed into a 9cm Petri dish. Each plate was then inoculated with a 4mm plug obtained from the margin of a 2 ~ 4d CMASS plate culture. After 2d incubation at 25℃, an aliquot of 10mL of 95% ethanol was added to each dish and maintained overnight under the same conditions[14]. Fungal isolates that grew well and developed large clear zones around the colonies after ethanol treatment were selected for further study on their xylanase production in liquid cultures.

1.3 Liquid cultures of selected fungal isolates

Four isolates, namely CY2809, CY4786, CY5040 and HU5048, were selected from the preliminary screening experiment. They were further cultured in a basal medium consisting of 10.0g/L of birchwood xylan, 2.5g/L of yeast extract and 15.0 g/L of sea salts. The inoculum of the culture of each isolate was obtained from three 7mm mycelial plugs cut from the edges of the fungal colonies grown on CMASS plates for 2 ~ 4d and homogenized in 25mL distilled water. An aliquot of 0.2mL of the homogenate was pipetted into 50mL of the basal medium in a 250mL Erlenmeyer flask. The liquid culture was incubated at 25℃ and shaked at 100r/min for 2 ~ 7d. The culture filtrate from each culture was collected by centrifuging the culture at 4000r/min (Universal 32, Hittach, Germany) for 10min and assayed for xylanase activity and the content of xylose-equivalent reducing sugars (CXERS). Mycelial biomasses were harvested by repeatedly rinsing with distilled water after each centrifugation. Finally, the mycelial biomass was weighed after oven drying at 105℃ for 24h and subsequent storage in desiccators until constant weight was achieved.

1.4 Optimization of culture conditions

The isolate CY4786 selected as best was grown at different regimes of nitrogen source, salinity, temperature and pH while 1.0% (W/V) birchwood xylan was consistently used as a sole carbon source in the medium. The nitrogen sources used at 0.035% (W/V) were: yeast extract, tryptone, l-asparagine (Sigma), urea, ammonium sulfate, ammonium nitrate potassium nitrate, and potassium nitrite. For screening of the nitrogen sources, all media were prepared in a solution of sea salts at 15g/L at pH 7.0 adjusted with 0.05mol/L phosphate buffer. The liquid cultures were incubated at 25℃ for 6 ~ 8d. To determine the optimal salinity for xylanase production, the basal medium was prepared using a solution containing sea salts 0, 6, 12, 18, 24, 30 and 36g/L respectively and the cultures were incubated at 25℃ for 7d. The optimal temperature was determined by incubating the cultures for 7d at 15, 20, 25, 30, 35 and 40℃, respectively. A range of pH from 4.2 to 9.0 at 0.4 unit intervals was tested for the basal medium, which was adjusted to pH 4.2 ~ 7.8 with 0.05mol/L citrate-phosphate buffer and pH 7.4 ~ 9.0 with 0.05mol/L Tris buffer. The cultures for pH tests were also incubated at 25℃ for 7d. All the cultures were shaken at 100r/min during incubation.

1.5 Assays for xylanase activities

Xylanase activity was assayed by means of measuring the CXERS released from the xylan substrate[5, 15]. The reaction mixture contained 0.5mL of the appropriately diluted enzyme preparation (culture filtrate), 1.0mL 0.05mol/L phosphate buffer at pH 7.0 and 0.5mL 1.0% birchwood xylan. After incubation at 37℃ for 20min, the reaction was terminated by adding 3mL of 3, 5-dinitrosalicylic acid to the mixture. The reacted mixture was then boiled for 10min and cooled down to ambient temperature by adding 10mL distilled water. Absorbance was measured at 550nm (Genesys 5™ Spectrophotometer, Spectronic Instruments, Inc., NY, USA). A control with the xylan substrate and boiled enzyme preparation was used for all the triplicate assays. One unit of xylanase activity was defined as the amount of the enzyme capable of releasing reducing sugars equivalent to 1.0 nmol xylose from the birchwood xylan per minute under the specified conditions.

1.6 Features of the enzyme preparation

The reaction temperature for the xylanase was evaluated by incubating the reaction mixtures at 20 ~ 90℃ at 10℃ intervals. The effect of pH on the reaction was assessed in 0.05mol/L citrate-phosphate buffer for pH 4.2 ~ 7.8 and 0.05mol/L Tris buffer for pH 7.4 ~ 9.0. To assess thermal stability, the enzyme preparation was incubated at 50, 60 and 70℃ for 60min and sampled at 15min intervals for assays after cooling on ice.

All chemicals used for the experiments described above were of analytical grade.

2 Results

2.1 Selected isolates

Among the 77 fungal isolates from the mangroves, only seven developed visible clear zones (15.5 ~ 27.5mm in diameter) around their colonies on CMASS plates after 2d incubation at 25℃. After ethanol treatment, clear zones appeared around the colonies of 34 isolates. The remaining 43 isolates did not exhibit any clear zones and these were considered to be unable to produce detectable xylanase activity. Based on the sizes of colonies and the clear zones, three isolates derived from mangrove decaying wood (CY2809) (*Staganospora* sp.), leaf debris (CY4786) and soil (CY5040) were chosen for further evaluation in parallel with HU5048 (*A. awamori*) (Table 1). The two isolates, CY4786 and CY5040, cannot be identified to specific taxa because both isolates do not produce any spores for identification though their vegetative growth were rapid on CMASS plates or in liquid cultures.

Table 1 The colony and clear zone sizes of selected fungal isolates derived from mangrove substrates after 2d incubation on CMASS plates at 25℃

Fungal isolates	Fungal species	Mangrove source	Colony diameter ± SD/mm	Clear zone diameter ± SD/mm	
				Before ethanol treatment	After ethanol treatment
CY2809	*Staganospora* sp.	Decaying wood	14.5 ± 0.9 c	20.7 ± 1.2 b	24.0 ± 0.9 c
CY4786	Unidentified	Leaf debris	13.5 ± 0.5 c	27.5 ± 0.9 a	30.0 ± 1.3 b
CY5040	Unidentified	Soil under debris	19.5 ± 0.5 b	21.0 ± 0.9 b	30.0 ± 1.8 b
HU5048	*Aspergillus awamori*	(Terrestrial soil)	36.0 ± 0.9 a	Not visible	41.0 ± 1.7 a

Means with different lowercase letters in each column differed significantly in Tukey's HSD test ($P < 0.05$).

The mycelial biomass, xylanase activity and CXERS measurements from the liquid cultures of the four isolates during 7d incubation at 25℃ are shown in Fig. 1. The three estimates for CY4786 reached 4.6mg/mL, 4370 unit/mL and 638μg/mL on day 2 respectively. The xylanase activity therein increased to 7368 unit/mL on day 7 while the CXERS gradually decreased to 413μg/mL and the biomass was maintained around 4.5mg/mL. For CY2809 and CY5040, maximal xylanase activities (2918 and 4133unit/mL) were achieved on day 7 and 5 whereas the CXERS peaked on day 3 (2073 and 1554μg/mL) respectively. As a positive control isolate for comparison, HU5048 (*A. awamori*) had maximal xylanase activity on day 7 (7697unit/mL), CXERS on day 3 (2386μg/mL), and mycelial biomass on day 5 (6.2mg/mL). Overall means of the xylanase activities on day 2 ~ 7 were 6247, 4019, 2213 and 1478unit/mL for CY4786, HU5048, CY5040 and CY2809 respectively, significantly differing from one isolate to another ($F_{3, 46} = 49846$, $P < 0.001$) or among the incubation periods ($F_{5, 46} = 24214$, $P < 0.001$). Thus, the isolate CY4786 was chosen as best for optimizing xylanase production conditions due to its excellent culture features and consistently high xylanase activities during the incubation period.

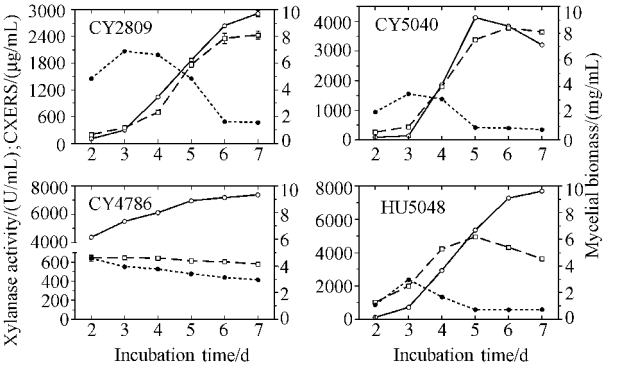

Fig.1 Trends in mycelial biomasses (□), xylanase activities (○), and CXERS (●) of the selected fungal isolates CY2809 (*Staganospora* sp.), CY4786, CY5040 and HU5048 (*Aspergillus awamori*) in the basal liquid medium during 7d incubation at 25℃. Visible or invisible error bars: SD.

2.2 Optimized conditions for xylanase production

The different nitrogen sources in the basal medium could greatly affect the xylanase production by CY4786 (Table 2). Inclusion of ammonium sulfate, yeast extract or l-asparagine led to significantly greater xylanase activities on day 6 ($F_{7, 14} = 913$, $P < 0.001$) and day 8 ($F_{7, 14} = 1205$, $P < 0.001$) compared with other nitrogen sources, of which potassium nitrate was least capable. This indicates that the isolate was able to utilize organic NH_2^- or inorganic NH_2^+, NH_4^+, NO_3^-

Table 2 Impact of different nitrogen sources on CXERS, mycelial biomass (MB) and xylanase activity (XA) of the mangrove-derived isolate CY4786 in the basal liquid medium containing 1% (W/V) birchwood xylan as a sole carbon source and 1.5% (W/V) sea salts incubated at 25℃ and 100r/min for 6 ~ 8d

Nitrogen source	CXERS/(μg/mL)		pH in filtrate		MB/(mg/mL)		XA/(U/mL)	
	Day 6	Day 8	Day 6	Day 8	Day 6	Day 8	Day 6	Day 8
Yeast extract	327.0 b	303.6 bcd	6.95 e	6.84 e	5.46 d	5.36 a	6682 b	6556 a
Tryptone	361.2 a	336.0 a	6.92 e	6.85 e	6.26 a	5.61 a	6265 c	5100 e
l-asparagine	310.8 b	289.2 d	7.32 a	7.43 b	5.84 bc	5.31 a	6837 a	6531 a
Urea	314.4 b	309.0 bc	7.02 d	7.13 d	5.24 de	5.15 ab	5526 d	6198 b
Ammonium sulfate	332.4 ab	321.6 ab	6.32 g	6.24 f	5.48 d	4.70 bc	6945 a	6090 c
Ammonium nitrate	309.0 b	300.0 cd	6.42 f	6.29 f	6.00 ab	5.15 ab	5311 e	6131 bc
Potassium nitrate	323.4 b	309.0 bc	7.10 c	7.22 c	4.95 e	4.44 c	2782 g	2887 f
Potassium nitrite	321.6 b	307.2 bcd	7.25 b	7.59 a	5.55 cd	4.57 c	4762 f	5521 d

Means with different lowercase letters in each column differed significantly in Tukey's HSD test ($P < 0.05$).

and NO_2^-. Among the inorganic nitrogen sources, NH_4^+ appears to be better for the xylanase production compared with NO_2^- or NO_3^-. Irrespective of using 0.05mol/L phosphate buffer at pH 7.0 in the basal medium, the pH values of the crude enzyme solutions from the cultures differed significantly among the nitrogen sources tested. Lower pH measurements resulted from the use of yeast extract, tryptone, ammonium sulfate or ammonium nitrate in the medium compared with that of l-asparagine, urea, potassium nitrate or potassium nitrite.

The medium salinities adjusted with sea salts in a range of 0 ~ 36g/L had significant effect on the production of xylanase by CY4786 ($F_{6,12} = 206$, $P < 0.001$). The optimal production was achieved at the salinity level of 12 ~ 18g/L (Fig.2-A), which is in accordance with salinity levels in the mangrove ecosystem from which the fungal isolates were derived. However, the xylanase production was significantly suppressed when the salinity was beyond the range.

The optimal temperature was determined to be around 25℃ (Fig. 2-B). Below or above this temperature, the production of xylanase by CY4786 in the cultures was largely reduced ($F_{4,8} = 339$, $P < 0.001$).

Initial pH levels in the basal medium also conspicuously affected the xylanase production. At pH 4.2 ~ 7.8 adjusted with 0.05mol/L citrate-phosphate buffer, the enzyme production tend to increase with increasing pH and peaked at pH 7.8 (Fig.2-C). The same trend was also observed at pH 7.4 ~ 9.0 adjusted with 0.05mol/L Tris buffer but the maximum activity recorded at pH 9.0 was lower than that at pH 7.8 adjusted with 0.05mol/L citrate-phosphate buffer. Apparently, the two different buffer systems varied in their effect on the xylanase production at the overlapping range of pH 7.4 ~ 7.8. Within this pH range, the Tris buffer apparently was not so effective to facilitate the enzyme production as citrate-phosphate buffer (relative activities: 81.2% ~ 84.5% versus 89.5% ~ 100%). Thus, the slightly alkaline culture conditions were suitable for the enzyme production by CY4786.

Based on the optimal culture conditions determined as above, the isolate CY4786 was cultured at the regime of 25℃ and 100r/min for 7d in the liquid medium consisting of birchwood xylan at 10.0g/L, yeast extract at 2.5g/L and sea salts at 15.0g/L with an initial pH of 7.8, and the resultant culture had high xylanase activity up to 1.07×10^4 unit/mL when assayed at pH 4.6 and 50℃.

2.3 Optimized assay conditions for xylanase activity

The xylanase produced by CY4786 under the optimized

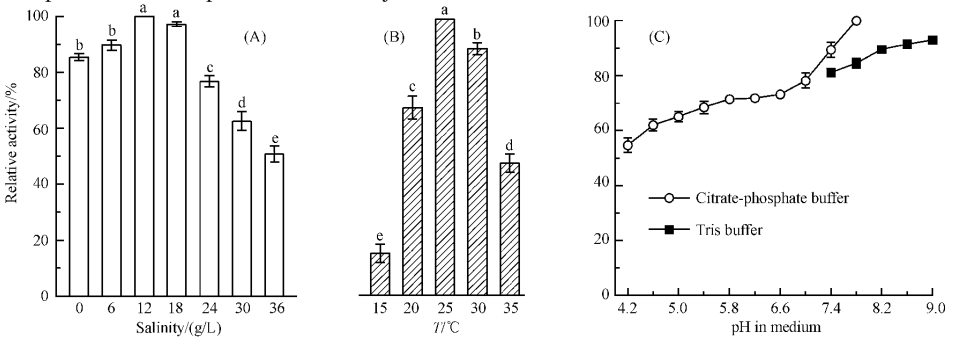

Fig. 2 Optimization of salinity (A), temperature (B) and pH (C), adjusted with the two buffer systems, for production of xylanase by the mangrove-derived fungal isolate CY4786 grown in liquid medium. Differences in heights of the bars with different lowercase letters were significant in Tukey's HSD test ($P < 0.05$). Error bars: SD.

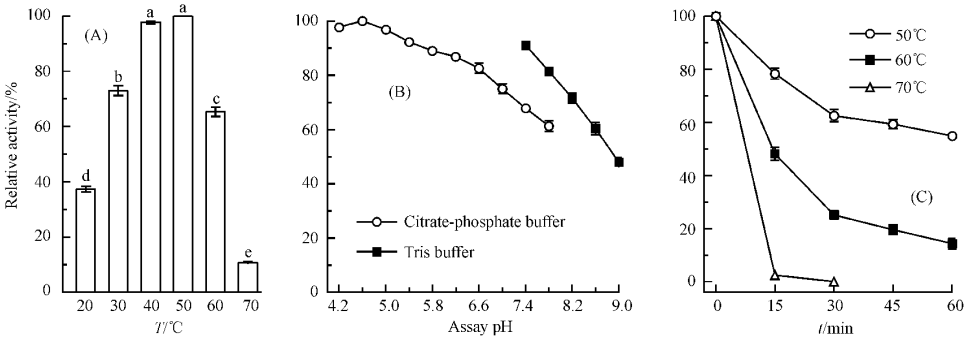

Fig. 3 Optimization of temperature (A) and pH (B) for assaying the activity of xylanase produced by the mangrove-derived fungal isolate CY4786 grown under optimized culture conditions and thermal stability of the enzyme at 50 ~ 70℃ (C). Differences in heights of the bars with different lowercase letters were significant in Tukey's HSD test ($P < 0.05$). Error bars: SD.

culture conditions as above was most active when assayed at 40 ~ 50℃ (Fig. 3-A) and pH 4.2 ~ 5.0 (Fig. 3-B). Significant decline in its activity was observed when the assay temperature was out of that range ($F_{5,10} = 899$, $P < 0.001$) or beyond pH 5.0 ($F_{9,18} = 314$, $P < 0.001$). Interestingly, the pH effect on the xylanase activity also differed between the two buffer systems in the overlapping range of pH 7.4 ~ 7.8, at which the Tris buffer resulted in higher activities compared with the citrate-phosphate buffer. On the other hand, the enzyme was intolerable to higher temperatures (Fig. 3-C). The loss of its activity was 45% at 50℃ and 86% at 60℃ during a 60min period of incubation but up to 97.6% within 15 min at 70℃.

3 Discussion

Based on the results presented above, a large portion of the fungal isolates derived from decaying wood, debris and sediment in the mangrove ecosystem in Hong Kong exhibited xylanase activities. Those fungi can act as decomposers of the hemicelluloses rich materials in mangroves and can be considered as a source of fungal isolates to be searched for xylanase production for industrial exploitation. The isolate CY4786 selected from 77 isolates of the mangrove fungi was characterized with fast growth and high xylanase activity (1.07×10^4 unit/mL) under optimized culture conditions (Fig. 2). Its xylanase was most active at pH 4.2 ~ 5.0 and 40 ~ 50℃ (Fig.3). This pH and temperature range is close to that in digestive tracts of animals, indicating its potential for application. However, the xylanase produced by the isolate was unstable above 50℃.

The basal medium used for xylanase production included birchwood xylan as a sole carbon source throughout the study. Fungal growth during incubation was maintained by uptake of its hydrolyzed products such as xylose and/or other reducing sugars. Thus, the CXERS present in the medium may reflect a status of the fungal growth and a relative level of xylanase production in the cultures, in which four phases can be recognized (Fig. 1). In phase 1, the CXERS rapidly accumulated during the first 2 or 3d incubation, corresponding to relatively low, but increasing, levels of mycelial biomass and xylanase production by the selected isolates CY2809, CY5040 and HU5048. Phase 2 was characteristic with the occurrence of the CXERS peak, i.e., a critical point at which the amount of the CXERS released from hydrolysis of the birchwood xylan was equal to that taken by the fungal growth. It was followed by phase 3 at which the amount of the CXERS required for the accelerating fungal growth exceeded the amount released and thus dropped quickly. This phase varied in duration among the isolates and was terminated when the mycelial biomass reached a maximum 2 or 3d after the CXERS peak. In phase 4, the CXERS in the liquid culture became stable at low levels, and the increase of xylanase production slowed down or decreased, apparently due to exhaustion of the mere carbon supply, while the xylanase produced in the culture accumulated to a maximal level. An exception was the isolate CY4786 whose phases 1 and 2 were completed within the first 2d after incubation due to its fast growth and excellent culture features. Thus, the levels of xylanase and CXERS in liquid culture were highest and lowest in all the cultures on day 2, respectively.

The four phases recognized above may help to simplify the process of screening fungal isolates for xylanase production. It is much more convenient to assay the CXERS than the xylanase activity that was based on the use of the birchwood xylan as substrate and could be easily affected by the factors such as assay temperature and pH. There was a significant correlation of the xylanase activities to the CXERS in phases 2 ~ 4 for all the fungal cultures (CY2809: $r^2 = 0.92$, $F = 36.2$, $P < 0.01$; CY4786: $r^2 = 0.99$, $F = 200.1$, $P < 0.01$; CY5040: $r^2 = 0.87$, $F = 19.3$, $P = 0.02$; HU5048: $r^2 = 0.87$, $F = 20.9$, $P = 0.02$).

Obviously, the correct determination of phase 2 by easily monitoring the CXERS in the cultures would allow for preliminary assessment of the potential of xylanase production by a fungal isolate. The earlier the CXERS peak occurs, the better the isolate will be, as demonstrated by CY4786. For a selected isolate, the relationship between the two variables would allow for estimating the level of xylanase activity by quickly measuring the CXERS in the culture under optimized conditions.

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产木聚糖酶的沿海红树林真菌筛选及其培养与酶活测定条件优化

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摘 要:从香港海岸红树林分离到的 77 株真菌中有 34 株可产生木聚糖酶,从中选出 CY2809 (*Staganospora* sp.)、CY4786 和 CY5040 等 3 菌株与已知陆生产酶菌株 HU5048 (*Aspergillus awamori*)进行产木聚糖酶的比较研究。根据培养液中菌丝生物量、木聚糖酶活力和木糖等价还原糖含量等指标的测定,菌株 CY4786 在起始 pH 7.8 的木聚糖-酵母膏-海盐液体培养基中 25℃下震荡(100r/min)培养 7d 产酶最佳 粗酶液在 50℃和 pH 4.6 的优化条件下进行测定,木聚糖酶活力达到 1.07×10^4 U/mL。结果表明,红树林真菌起着半纤维素降解者的作用,沿海红树林环境中存在着可资利用的木聚糖酶产生菌。作者讨论了利用发酵液中木糖等价还原糖含量的动态变化作为快速筛选产木聚糖酶菌株的指标的可能性。

关键词 红树林真菌,木聚糖酶,还原糖,基本培养基,培养条件优化

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