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Full Length Research Paper

Efficient utilization of xylanase and lipase producing thermophilic marine actinomycetes (*Streptomyces albus* and *Streptomyces hygroscopicus*) in the production of ecofriendly alternative energy from waste

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The marine actinomycetes strain MAC 6 and MAC 14 were used in the production of xylanase and lipase enzyme using wheat bran as an inducer with oats, soy meal and ground nut oil cake. Two strains showed higher xylanase and lipase activity at pH 10.0 and 50°C among 30 actinomycetes screened from sediments of Tiruchendhur coastal areas of Tamil Nadu. The enzymes were produced by solid state fermentation by using 70% of moisture content. More biogas production was observed in oil cake and straw waste with the pretreatment of xylanase and lipase producing strains within short hydraulic retention time.

Key words: Biogas, groundnut oilcake, lipase, marine actinomycete, rice straw, solid state fermentation, thermophilic, wheat bran, xylanase.

INTRODUCTION

Marine actinomycetes are exploited for efficient enzyme production because of their extraordinary features of high thermophilic and alkaliphilic nature. Marine microbial enzymes are relatively more stable and active than corresponding enzymes derived from plants or animals (Kin, 2006; Bull et al., 2000). Mixed solid state fermentation (SSF) is better than submerged fermentation (SMF) because of low capital investment, reduced energy requirement and improved product recovery. Lipases and xylanases are the important commercial enzymes used in many fields. Lipases (triacyl glycerol acyl hydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long chain fatty acids. Lipases are used in detergent, food and agrochemical industries (Pallavi et al., 2010). The actinomycete *Streptomyces griseochro-mogenes* isolated from shrimp pond showed higher lipase activity (Gunalakshmi et al*.,* 2008). Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan. Actinomycetes strains (*Streptomyces roseiscleroticus)* secreting high activity xylanase, are thermotolerant in nature (Grabski and Jeffries, 1991). Thus, actinomycetes are an important group of microorganisms involved in lignocellulose degradation (McCarthy, 1987). Xylanases are used in paper industry for prebleaching process (Bajpai, 1999), in animal feed industry, to increase the body weight of the animals (Silversides and Bedford, 1999; Kung et al., 2000) and in bakery industry to increase the dough viscosity, bread volume and shelf life (Romanowska et al., 2003).

The continuous utilization of fossil fuels causes serious effects to the environment as global warming and air pollution. But bio energy is eco-friendly safe and does not cause any pollution effects. The energy from biomass

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holds a promising scope under Indian conditions because this sector encourages the efficient management of agricultural waste in an eco-friendly way (Stalin et al., 2012). Biogas production was observed from codigestion of cow dung with rice husk, coconut pith and rice chaff (Elijah et al., 2009; Radhika et al., 1983; Vivekanandan and Kamaraj, 2011).

MATERIALS AND METHODS

Isolation of actinomycetes from sediment sample

The actinomycetes were isolated from the marine sediment samples collected from two different points of Tiruchendur coastal areas of Tamil Nadu, India. The collected samples were immediately transported to the laboratory. The air dried samples were incubated at 55°C for 10 min and serially diluted. It was spread on actinomycetes isolation agar media with pH 9 and incubated for seven to 14 days. 20 mg/L nystatin and cycloheximide 100/L was added to control bacterial and fungal contaminations. The pure actinomycetes culture was maintained on nutrient agar media for further analysis.

Screening of lipase and xylanase producing actinomycetes

The actinomycetes were grown on tributyrin agar plates and the zone of clearance was observed by the hydrolysis of tributyrin (Hun et al., 2003; Nair and Kumar, 2007). The actinomycetes isolates were tested to produce xylanase by growing them on xylan-agar medium (nutrient agar added with 2.5 g/L xylan) for two days. The plates were then stained with Congo red solution composed of 0.5% Congo red and 5% ethanol for 15 min and destained with 1 M Nacl. The xylanase producing isolates were screened by observing yellow zones around the colonies against the red background (Teater and Wood, 1982). The actinomycetes that showed the maximum zone of clearance was selected for further analysis.

Inoculum production

A loopful of cells from a freshly grown slant culture was inoculated into 100 ml of sterile modified mineral salt solution. The composition of media (grams/100 ml) was as follows: magnesium sulphate, 0.05 g; dipottasium hydrogen phosphate, 0.1 g; sodium chloride, 4 g; ferrous sulphate, 0.001 g; manganous chloride, 0.001 g; zinc sulphate, 0.001 g. It was incubated at 50°C in an incubator shaker at 180 rpm for 24 h.

Solid state fermentation

The different wastes with the following combinations were used for enzyme production. For lipase production: substrate 1, groundnut oil cake (10 g); substrate 2, soy meal + groundnut oil cake (5 + 5 g) and substrate 3, groundnut oil cake (3.3 g) + soy meal (3.4 g) + wheat bran (3.3 g). For xylanase production: substrate 1, wheat bran (10 g); 2, soy meal + wheat bran (5 + 5 g); 3, wheat bran (3.3 g) + soy meal (3.4 g) + oats (3.3 g).The waste substrates were dried at room temperature to reduce the moisture content and ground to the desired size. Each substrate (1, 2 and 3) of 10 g was added with 80 ml of modified mineral salt solution, autoclaved. After cooling, 10 ml of inoculum was added to the modified mineral salt media with solid waste as a substrate. Each flask with substrate 1, 2 and 3 was incubated at 50°C in an incubator shaker at 180 rpm for eight days.

Optimization of different parameters and enzyme extraction

All the experiments were carried out in 250 ml Erlenmeyer flask containing 100 ml of medium with the desired substrate. It was incubated for eight days at 50°C in an incubator shaker at 180 rpm. The different parameters like pH of the medium (6, 7, 8, 9, 10 and 11), temperature (35, 40, 45, 50, 55 and 60°C), moisture content (50, 60, 70, 80, 90 and 100%), substrate concentration (5, 10 and 15%), sodium chloride concentration (1, 2, 3, 4 and 5%) were optimized for both lipase and xylanase enzyme production. The crude enzyme from the fermented substrate was extracted by sodium phosphate buffer (pH 9.0). The fermented substrate was mixed with 100 ml of buffer and kept in the rotary shaker (180 rpm) at 50°C for 1 h. The raw extract was obtained by pressing the mixture and subsequent centrifugation at 10000 rpm for 15 min at 4°C. The clear supernatant obtained from centrifugation was used to determine enzyme activity.

Lipase and xylanase assay

Lipase activity was measured by titrimetric method using olive oil emulsion method (Watnabe et al., 1977). The reaction mixture with 5 ml of olive oil emulsion (25 ml olive oil and 75 ml 2% polyvinyl alcohol), 4 ml of 0.2 M tris buffer, 1 ml of 110 mM $CaCl_2$ and 1 ml enzyme solution was incubated for 30 min at 55°C. The control containing boiled inactivated enzyme (at 100°C for 5 min) was treated similarly. After incubation, the enzyme activity was blocked by 20 ml of acetone and ethanol (1:1) mixture and the liberated free fatty acid was titrated against 0.05 M NaOH using phenolphthalein as an indicator. One unit of lipase was defined as the amount of enzyme that liberates 1 µmol of fatty acid/min under standard assay conditions.

Xylanase activity was assayed by incubating 1.0 ml of enzyme extract with each 1 ml of 1% xylan and 4 ml of sodium phosphate buffer (pH 9.0) at 50°C for 30 min. A control without enzyme was used in this assay. Released reducing sugar was measured by dinitrosalicylic acid (DNS) method (Miller, 1959). To this mixture, 3 ml of DNS reagent was added and heated in a boiling water bath for 10 min. At the time of cooling, 1 ml of freshly prepared 40% sodium potassium tartarate solution was added and the samples were read at 510 nm in U.V. spectrophotometer. The enzyme activity was expressed as IU/ml.

Partial purification of enzymes

The crude enzyme was precipitated by addition of 60% (v/v) volume of chilled acetone and stored overnight at -4°C. It was centrifuged at 10000 rpm for 10 min. The precipitate was suspended in sodium phosphate buffer and incubated overnight at 4°C. The enzyme was dialyzed against same phosphate buffer. It was loaded on column (2.5 × 17 cm) of diethylaminoethanol (DEAE) Sephadex A-50 already equilibrated with sodium phosphate buffer. The purified enzyme was collected and stored at 4°C for further use.

Utilization of xylanase and lipase producing marine *Streptomyces* in biogas production

Four sets of batch digesters were taken. In digesters 1 and 2, rice straw was chopped into pieces and mixed with 100 ml of water and incubated overnight at 90°C. In digesters 3 and 4, groundnut oilcake was taken. In sets 2 and 4, 10 ml of xylanase and lipase producing strains were added as inoculum and incubated for Seven days. To all the digesters, 200 g of cow dung, 400 ml of sterile water and 100 ml of digested slurry were added. Sets 1 and 3 were used as control. The digesters were allowed for biomethanation at

Table 1. Influence of different pH and temperature on xylanase and lipase activity.

pH	Enzyme activity (IU/ml/min)		Temperature (°C)	Enzyme activity (IU/ml/min)	
	S. albus (Xylanase)	*S. hygroscopicus* (Lipase)		*S. albus* (xylanase)	*S. hygroscopicus* (lipase)
6	90.17±0.11	58.49±0.17	35	201.12±0.46	109.49±0.30
7	181.45±0.24	87.65±0.13	40	208.82±0.65	187.37±0.13
8	257.70±0.09	179.55±0.31	45	289.37±0.18	199.8±0.26
9	276.42±0.26	204.66±0.40	50	302.50±0.36	241.64±0.29
10	302.50±0.36	241.64±0.29	55	279.59±0.33	204.52±0.30
11	273.39±0.19	188.43±0.93	60	263.19±0.86	158.37±0.27

Table 2. Influence of different substrate concentration and sodium chloride tolerance on xylanase and lipase activity.

Substrate concentration (%)	Enzyme activity (IU/ml/min)		Sodium chloride (%)	Enzyme activity (IU/ml/min)	
	S. albus	*S. hygroscopicus*		*S. albus*	*S. hygroscopicus*
5	181.15±0.05	112.58±0.36	1	154.16±0.58	104.58±0.34
10	302.50±0.36	241.64±0.29	2	237.58±0.21	145.49±0.37
15	191.67±0.17	166.36±0.25	3	282.74±0.16	204.52±0.29
			4	302.50±0.36	241.64±0.29
			5	276.41±0.09	200.34±0.78

35 ± 5°C with pH 7.5±5 as a batch process over a period of 30 days hydraulic retention time (HRT). Strictly anaerobic condition was given to this set up. Biogas production was measured daily on volume basis by water displacement method (Baba et al., 2012).

RESULTS AND DISCUSSION

Screening and identification of potential isolate for lipase and xylanase activity

By larger clear zone formation on xylan and tributyrin agar medium, the marine actinomycete MAC 6 and MAC 14 were screened as a potential isolate for xylanase and lipase activity. The organisms were identified as *Streptomyces albus and Streptomyces hygroscopicus* by 16S rRNA gene partial sequencing method (GENBANK accession number for nucleotide sequence: JQ801296 and JQ801299).

Influence of pH and temperature on lipase and xylanase activity

pH is an important parameter for growth of microbes in specific media. The enzyme activity was highly influenced with the change of pH. The lipase and xylanase producing *Streptomyces* species showed highest enzyme activity at alkaline pH 10.0 and 50°C (Table 1). The highest value for lipase and xylanase were 241.64 ± 0.29 and 302.50±0.36 IU/ml/min. The lipase and xylanase producing *S. hygroscopicus* and *S. albus* exhibited maximum activities from pH range of 9 to 11. It showed reduced enzyme activities for pH 6 to 7. The results from Table 1 show that marine actinomycetes preferred more alkaline conditions. Both strains exhibited maximum enzyme activity at 50°C. *S. albus* showed maximum xylanase activity at 45 and 50°C and *S. hygroscopicus* showed highest lipase activity at 50 and 55°C. But the enzyme activity was reduced at 35 and 40°C. From Table 1, it was observed that both xylanase and lipase activity was high at 45 to 55°C. Hala et al. (2010) reported that the highest lipase activity for *Fusarium* species was at pH 9.5. The highest lipase activity for marine actinomycetes at pH 8 and 55°C was observed by Gunalakshmi et al. (2008). Maximum xylanase production was observed by Rajashri and Anandrao (2012) at pH 8 and 45°C for wheat bran as substrate in solid state fermentation. Sevanan et al. (2011) reported that the highest xylanase activity was at pH 9. The results indicate the high alkaliphilic and thermophilic feature of marine actinomycetes.

Influence of different substrate concentration and sodium chloride tolerance on lipase and xylanase activity

Both strains showed highest xylanase and lipase activity at 10% substrate concentration (Table 2). The enzyme activity was reduced at 5% concentration (xylanase- 181.15 ± 0.05 IU/ml/min, lipase- 112.58 ± 0.36 IU/ml/min). The xylanase and lipase production was reduced at high concentration of the substrate because of reduced penetration of the organism and reduced mass transfer rate (Rao et al., 2003). The substrate

Table 3. Influence of different moisture content on xylanase and lipase activity.

Moisture content (%)	Enzyme activity (IU/ml/min)	
	S. albus	***S. hygroscopicus***
50	105.00±0.30	41.64±0.12
60	227.13±0.44	158.74±0.22
70	302.50±0.36	241.64±0.29
80	276.41±0.13	179.46±0.24
90	211.57±0.17	129.42±0.24
100	74.81±0.18	58.57±0.31

Table 4. Influence of inducers with waste on xylanase activity.

Agro industrial waste	***S.albus*** **(IU/ml/min)**
Wheat bran	185.29±0.35
Wheat bran + soya	231.67±0.49
wheat bran + soya + oats	302.50±0.36

concentration of 10 g had showed maximum xylanase and lipase production when compare with 5 and 15 g owing to easier penetration by microbes.

Both strains showed highest activity at 4% sodium concentration. The maximum activity was also observed at 3% concentration (xylanase- 282.74 ± 0.16 IU/ml/min, lipase- 204.52 ± 0.29 IU/ml/min). From Table 2, it was noted that both the strains showed least activity at 1% concentration for xylanase and lipase enzymes. Gunalakshmi et al. (2008) reported that 4% sodium chloride concentration was favourable for highest lipase activity by marine actinomycetes. It confirmed the high sodium chloride tolerance and high alkaliphilic feature of marine actinomycetes.

Effect of moisture content on xylanase and lipase activity

Table 3 shows the variations in moisture content that affected enzyme activity. Moisture content is an important parameter in solid state fermentation. From Table 3, it was observed that the maximum xylanase (302.50 ± 0.36) and lipase production (241.64 ± 0.29) was at 70% moisture content for the both species. The production of xylanase was reduced when the level of moisture was decreased to 50%. The insufficient moisture leads to the reduction of solubility of nutrients in media. Lonsane et al. (1985) reported that the high moisture content decreased the porosity and results in stickiness and also increased the contamination rate. Manoj et al. (2010) observed that the highest lipase activity was at 70% moisture content. According to Sarat et al. (2010), 60% moisture content was favourable for lipase production. Gaurav et al. (2010) observed that the highest xylanase activity was at 80% moisture content. The observations from Table 3 shows that 60 to 80% moisture content range was favourable for highest lipase and xylanase production but the highest was at 70%.

Optimization of waste with inducers

From Table 4, it was observed that xylanase activity was maximum (302.50 + 0.36 IU/ml/min) in the mixed wheat bran with soy meal and oats when compared with xylanase activity (231.67 + 0.49 IU/ml/min) observed in wheat bran with soy meal and (185.29 + 0.35 IU/ml/min) wheat bran as a substrate. The results showed that soy meal and oats act as inducers for the xylanase production and the combination of both with wheat bran increased the yield of xylanase production. Table 5 shows the influence of ground nut oil cake with inducers in lipase production. The single waste (groundnut oil cake) showed reduced enzyme production (150.23 ± 0.22 IU/ml/min) when compared with combination of soy meal and wheat bran. The combination of both inducers with groundnut oil cake (soya and wheat bran) increased the enzyme production (241.64 ± 0.29 IU/ml/min). Imandi and Garapathi et al. (2007) reported that lipase activity was high in the combination of wheat bran and bagasse as substrate. The observations from Tables 4 and 5 confirm that the addition of inducers (soy meal, wheat bran) with waste enhanced lipase and xylanase production rate.

Biogas production from oilcake and straw waste by pretreatment with *S. hygroscopicus* and *S. albus*

Tables 6 and 7 show the results for biogas production using oilcake and rice straw waste with predigestion of *S. hygroscopicus* and *S. albus*, respectively. The biogas

Table 5. Influence of inducers with waste on lipase activity.

Agro industrial waste	*S. hygroscopicus* (IU/ml/min)
Ground nut oilcake	150.23±0.22
Ground nut oilcake + soya	204.39±0.20
Groundnut oil cake + soya + wheat bran	241.64±0.29

Table 6. Biogas production using oilcake waste with pretreatment by *S. hygroscopicus*.

Days	Control	Biogas production (ml)
2	63.83 ± 6.44	126.0 ± 5.56
5	130.80 ± 7.71	295.6 ± 9.60
10	171.50 ± 8.04	405.16 ± 6.75
15	200.36 ± 6.35	533.73 ± 7.65
20	333.16 ± 9.25	686.13 ± 8.35
25	167.76 ± 4.14	480.8 ± 4.25
30	117.53 ± 4.96	193.33 ± 5.85

Table 7. Biogas production using rice straw waste with pretreatment by *S. albus*.

Days	Control	Biogas production (ml)
2	44.80 ± 7.34	85.83 ± 5.79
5	106.93 ± 5.00	153.56± 7.25
10	142.10 ± 4.29	214.6 ± 4.13
15	187.50 ± 5.26	313.46 ± 4.38
20	246.66 ± 4.50	483.23 ± 7.60
25	142.83 ± 6.29	303.93 ± 6.66
30	92.9 ± 5.16	139.13 ± 6.34

*Values are based on mean ± SD of 3 individual observations.

production was observed for 30 days hydraulic retention time (HRT). In the first interval (2 to 10 days), the biogas production was slightly increased. In the second interval (11 to 20 days), the gas production reached the peak rate. In the third interval, the rate of gas production decreased gradually in both samples. When compared with the control, the waste with pretreatment of lipolytic and xylanolytic strains showed high biogas rate on the 20th day in oilcake sample (686.13 ± 8.35 ml) and in straw waste (483.23 ± 7.60 ml). The high viscosity of the medium was responsible for the reduction of gas rate in both the control with oilcake and straw waste. But the samples treated with *S. hygroscopicus* and *S. albus* showed increased biogas rate because of easier digestion and more reduction in viscosity of the sample. Oilcake waste with digestion of *S. hygroscopicus* produced more biogas than rice straw waste. The addition of cowdung slurry with oilcake and straw waste enhanced the activity of methanogenic bacteria and increased the rate of biogas production. Vivekanandan and Kamaraj (2011) observed that the combination of rice chaff and cow dung increased the biogas production. According to Elijah et al. (2009), codigestion of rice husk and cowdung showed enhanced biogas production on the 38th day of experiment. The present study shows the peak rate in the 20th day because of predigestion of samples with marine *Streptomyces* species. The results indicate that pretreatment of waste samples with marine *Streptomyces* increased the digestion rate of waste and reduced the viscosity level. This condition was favourable for methanogens to use the waste for methane production in the next stage and increased the rate of biogas production within a short period.

Conclusion

The waste was used as substrate in solid state fermentation for production of lipase and xylanase enzymes. It showed the efficient ecofriendly management

of waste for enzyme production. The waste with inducers as wheat bran, soy meal and oats showed highest xylanase and lipase production when compared with single waste as substrate. Both species preferred alkaline pH 10 and higher temperature of 50°C for maximum xylanase and lipase production. The results indicate that waste with pretreatment of lipolytic and xylanolytic strains increased the digestivity of lignocellulosic and lipid enriched oil cake waste and enhanced the yield of biogas production when compared with the control. It showed the utility of xylanase and lipase producing organisms as an enhancer in biogas production and increased the yield of biogas. This method showed a good pathway for the efficient management of waste for the production of ecofriendly alternative energy.

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