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Sugar cane bagasse pretreatment: An attempt to enhance the production potential of cellulases by *Humicola insolens* TAS-13

Ikram-Ul HAQ, Muhammad Mohsin JAVED* and Tehmina Saleem KHAN

Institute of Industrial Biotechnology, GC University, Lahore Pakistan

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Abstract

Pretreatment of the cellulosic substrate has miracle effect on the enhancement of cellulase production by fungal strains. A thermophilic strain of *Humicola insolens* TAS-13 was locally isolated and was tested for cellulases production under solid-state fermentation conditions using sugar cane bagasse as substrate. The cultural conditions for the *H. insolens* were also optimized for the higher rate of cellulase secretion. In order to enhance the production rate of heterogenous cellulosic proteins, bagasse was pretreated with NaOH, H_2SO_4, H_2O_2 and H_2O_2+1.5%NaOH. The pretreatment of bagasse with 2.0% H_2O_2 along with 1.5% NaOH enhanced the biosynthesis of cellulases by *H. insolens*. Production rate was also optimized with different parameters like thickness of fermentation medium, initial pH, incubation time and temperature. The thickness of the fermentation medium of 0.8 cm (10 g) with pH range of 5.5 was found to be better for enhanced production at 50°C. The yield of the enzyme was reached maximum with CMC-ase (18.98 U/g/min), FP-ase (13.63 U/g/min), β-glucosidase (19.54 U/g/min) 72 h after inoculation.

Keywords: CMC-ase, FP-ase, β-glucosidase, solid-state fermentation

*Author to whom correspondence should be addressed. **E-mail**: mmj_bot@yahoo.com

INTRODUCTION

Cellulose is the most abundant organic source of food, fuel and chemicals however; its usefulness for the industrial approach is dependent upon its hydrolysis to glucose[1]. Fungi can be cultivated in a relative short time by establishing the methods of fermentation to produce a regular supply of cellulases[2]. Solid-state fermentation involves the growth of microorganisms on moist substrate. It offers advantages over liquid fermentation, as there is higher productivity, reduced energy requirements, low capital investment, low waste water out put, higher concentration of metabolites obtained and low downstream processing cost[3]. Sugarcane bagasse is abundantly and cheaply available as a byproduct from sugar industry. Direct use of sugarcane bagasse is not susceptible to exploit as growth substrate for cellulase production, therefore pretreatment was undertaken to study its potential in cellulase production by thermophilic fungal strain *Humicola insolens* TAS-13.

MATERIALS AND METHODS

Organism

A thermophilic strain of *Humicola insolens* TAS-13 was locally isolated by plate screening method as described by Clark *et al*,[4]. This screening test was based upon the zone formation as produced by cellulose hydrolysis. The isolated strain was then maintained (45°C) on cellulose agar medium for further use and stored at 4±°C.

Pretreatment of substrate

Dried sugar cane bagasse was grinded in an electric grinder to attain 0.5 mm size of mesh powder and then treated with different concentrations of NaOH, H_2SO_4, H_2O_2 (35%) or H_2O_2+1.5% NaOH separately, with 1:10 (v/w) ratio in an autoclave at 121ºC for 15 min. After treatment, all the samples were washed with distilled water to neutralize the effects of chemicals and dried in an oven at 80ºC for 12 h.

Inoculum preparation and fermentation

A loop full of conidia from 4-6 days old slant culture of *H. insolens* were aseptically transferred to a cotton wool plugged Erlenmeyer conical flask containing 50 ml of sterilized mineral salt cellulose medium (g/L; 1.4 $(NH_4)_2SO_4$, 2.0 KH_2PO_4, 0.3 urea, 0.3 $MgSO_4.7H_2O$, 0.0014 $ZnSO_4.7H_2O$, 0.005 $FeSO_4.7H_2O$, 0.0016 $MnSO_4$, 0.002 $CoCl_2$, 0.002 $CaCl_2$, 2.0 ml Tween-80 and 1.0 poly-peptone). The flask was incubated at 45°C on a rotary shaking incubator for 24 h and the freshly grown mycelial suspension was used as vegetative inoculum. Pretreated bagasse (15 g) was transferred to 250 ml conical flasks, which were incubated at 45ºC for 72 h after moistened the bagasse by adding 20 ml of mineral salts medium (pH 5.0) and 2.0% (v/w) vegetative inoculum. The flasks were shaken twice a day till the end of fermentation period.

Saccharogenic determination

Carboxymethyl cellulase (CMC-ase) was determined by the method of Wood and Bhat[5] using carboxymethyl cellulose (CMC) as substrate. Filter paper-cellulase (FP-ase) activity was measured by the method as described by Mandels and Sternberg[6] after filter paper hydrolysis and β-glucosidase was estimated using *p*-nitrophenyl-β-D-glucopyranoside (pNPNG) according to the method used by Rajoka and Malik[7]. The reducing sugars, released in case of CMC-ase and FP-ase were measured by standard dinitrosalicylic acid method[8].

Statistical analysis

Treatment effects were compared after Snedecor and Cochrane[9] using computer software Costat, cs6204W.exe. Significance difference among replicates has been presented as Duncan's multiple range tests in the form of probability <*p*> values.

RESULTS AND DISCUSSION

In the presented work, sugar cane bagasse was used as substrate under the solid-state fermentation conditions. Bagasse pretreatment was carried out with different concentrations of NaOH, H_2SO_4, H_2O_2 and H_2O_2+1.5%NaOH (Table 1). It was observed that best results were obtained, when bagasse was treated with 2.0% H_2O_2+1.5 NaOH, which gave higher yield of CMC-ase (13.69 U/g/min), FP-ase (9.16 U/g/min) and β-glucosidase (12.14 U/g/min).

Table 1: Pretreatment of bagasse for the enhance production of cellulases by *H. insolens* TAS-13

Reagents	Concentration (%) on the weight of substrate	Enzyme activity (U/g/min)		
		CMC-ase	FP-ase	β-glucosidase
	Control	10.72 ± 0.06^{defg}	5.89 ± 0.07^{defg}	10.07 ± 0.08^{defg}
NaOH	1.0	10.72 ± 0.07^{defg}	5.91 ± 0.09^{cdefg}	10.08 ± 0.09^{defg}
	2.0	10.78 ± 0.08^{defg}	5.94 ± 0.07^{bcdef}	10.09 ± 0.07^{defg}
	3.0	10.84 ± 0.09^{defg}	5.97 ± 0.08^{bcdef}	10.11 ± 0.08^{defg}
	4.0	10.56 ± 0.11^{ghi}	5.82 ± 0.05^{defg}	9.97 ± 0.07^{ghi}
	5.0	10.24 ± 0.12^{ij}	5.64 ± 0.05^{fg}	9.66 ± 0.08^{ij}
H_2SO_4	1.0	10.84 ± 0.13^{def}	5.96 ± 0.06^{bcdef}	10.14 ± 0.01^{def}
	2.0	10.92 ± 0.14^{ghi}	6.02 ± 0.06^{cde}	10.22 ± 0.02^{ghi}
	3.0	10.50 ± 0.15^{hij}	5.78 ± 0.07^{defg}	9.83 ± 0.04^{hij}
	4.0	10.36 ± 0.07^{j}	5.71 ± 0.08^{efg}	9.69 ± 0.05^{j}
	5.0	10.16 ± 0.06^{efg}	5.60 ± 0.09^{g}	9.51 ± 0.05^{efg}
H_2O_2	1.0	10.70 ± 0.06^{cde}	5.89 ± 0.07^{defg}	10.01 ± 0.04^{cde}
	2.0	11.04 ± 0.05^{cd}	6.08 ± 0.02^{bcd}	10.33 ± 0.02^{cd}
	3.0	11.06 ± 0.07^{ghi}	6.10 ± 0.01^{bcd}	10.36 ± 0.02^{ghi}
	4.0	10.55 ± 0.08^{ghi}	5.80 ± 0.01^{defg}	9.86 ± 0.01^{ghi}
	5.0	10.54 ± 0.07^{d}	5.82 ± 0.02^{defg}	9.85 ± 0.01^{b}
H_2O_2	1.0	11.39 ± 0.08^{b}	6.25 ± 0.02^{b}	10.62 ± 0.03^{b}
+	2.0	13.69 ± 0.07^{a}	9.16 ± 0.03^{a}	12.14 ± 0.04^{a}
1.5% NaOH	3.0	11.33 ± 0.07^{bc}	6.22 ± 0.04^{bc}	10.57 ± 0.05^{bc}
	4.0	10.84 ± 0.08^{efg}	5.97 ± 0.05^{bcdef}	10.14 ± 0.06^{efg}
	5.0	10.69 ± 0.05^{fgh}	5.89 ± 0.07^{cdefg}	10.01 ± 0.07^{fgh}
LSD		**0.2954**	**0.2812**	**0.2954**

Each value is an average of three replicates; ± denotes standard deviation among these replicates. Numbers followed by different letters differ significantly at P≥ 0.05

It was 21.70, 34.72 and 17.05% higher than that of control (without any pretreatment), respectively. It may be due to increase in the crystallinity and decrease in the lignin contents after pretreatment, so the substrate becomes more acceptable for the fungal strains. Effect of varying depth of wheat bran ranging 0.5-2.2 cm (5.0-25 g per flask) was investigated on the production of enzymes (Table 2). It was found that the production of CMC-ase, FP-ase and β-glucosidase (17.33, 12.01 and 16.27 U/g/min, respectively) were maximal at the substrate depth of 0.8 cm (10 g). As the depth was increased further, a significant loss in productivity was observed. *H. insolens* is an aerobic fungus and requires adequate supply of aeration. As the depth of the substrate was increased the metabolic pathway of the fungus effected, which reduce its extracellular cellulolytic efficiency[10].

Table 2: Effect of depth of substrate on the production of cellulases by *H. insolens* TAS-13

Substrate (gm/flask)	Depth (cm)	Enzyme activity (U/g/min)		
		CMC-ase	FP-ase	β-glucosidase
5.0	0.5	12.38±0.12^{e}	8.621±0.05^{e}	11.71±0.10^{c}
10	0.8	17.33±0.13^{a}	12.01±0.06^{a}	16.27±0.11^{a}
15	1.2	13.69±0.14^{b}	9.161±0.07^{b}	12.14±0.12^{b}
20	1.8	12.29±0.15^{d}	8.561±0.08^{d}	11.63±0.13^{d}
25	2.2	11.01±0.16^{e}	7.642±0.09^{e}	10.39±0.14^{e}
LSD		**0.01931**	**0.01931**	**0.01931**

Each value is an average of three replicates; ± denotes standard deviation among these replicates. Numbers followed by different letters differ significantly at P≥ 0.05

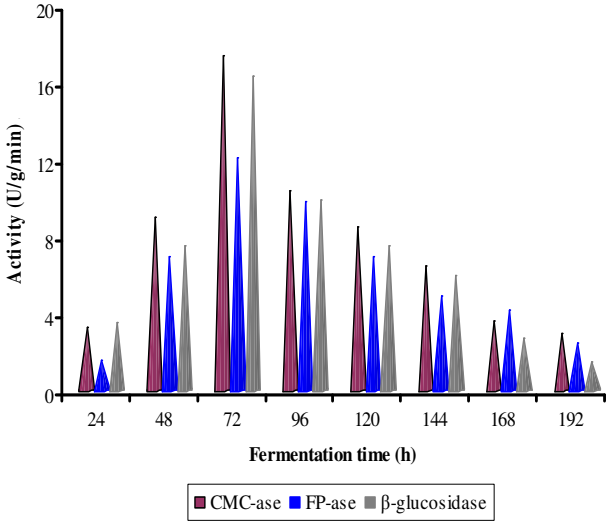

Fig. 1: Rate of cellulase production by *H. insolens* TAS-13 has shown by columns with pyramids

In the present study, the fermentation medium was incubated at 45°C for different time intervals (Fig. 1). The production of cellulolytic enzymes was reached maximum 72 h after inoculation. Further increase in the incubation period effects lethally. It might be due to the depletion of nutrients in substrate, which resulted in the inactivation of enzyme synthesis with the passage of time. Another reason is that initially the substance was more susceptible, which made rapid rise in enzyme synthesis. With the lapse of time, the susceptible portion was completely hydrolyzed to glucose, which severely inhibited the biosynthesis of cellulolytic enzymes[11,12,13].

The production of cellulases by *H. insolens* at different pH (3.0-8.0) of fermentation medium was also studied (Fig. 2). Results showed that higher rate of CMC-ase, FP-ase and β-glucosidase were obtained at slightly acidic (5.5) pH. A total of 6.53, 5.28 and 6.17% increase in CMC-ase, FP-ase and β-glucosidase was observed at this very pH. Further increase or decrease in pH results in the reduction of enzyme activity, which shows that the acidic pH is more favorable for the growth of *H. insolens* strain utilizing cellulosic biomass.

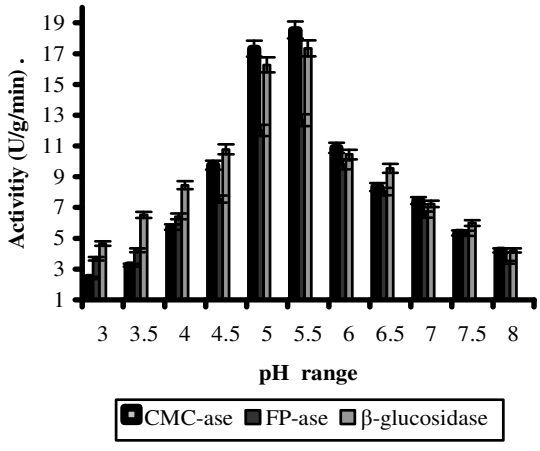

Fig. 2: Effect of hydrogen ion concentration (pH) of fermentation medium on the production of cellulases by *H. insolens* TAS-13

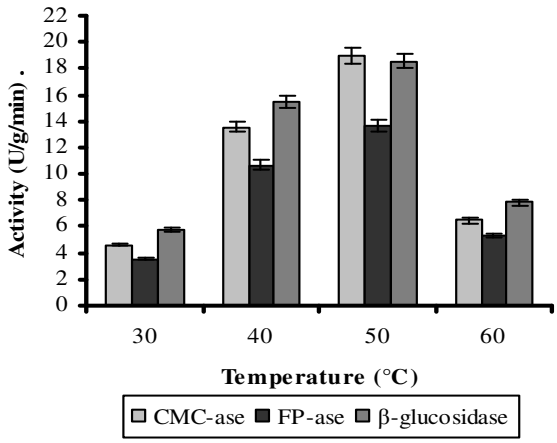

Fig. 3: Effect of fermentation temperature on the production of cellulases by *H. insolens* TAS-13

Fungal strain *H. insolens* was also grown under all the optimized conditions at different temperatures (30-60°C) in order to optimize the fermentation temperature for enhanced cellulase production (Fig. 3). It was found that enzyme secretion rate from the thermophilic strain was better at 50°C (18.98 U/g/min CMC-ase, 13.63 U/g/min FP-ase and 18.54 U/g/min β-glucosidase). Negative effects were observed on the production by increase or decrease in the temperature.

Bagasse is an agricultural byproduct; enriched with cellulosic biomass and is a preferable solid-substrate for the stimulation of cellulase enzymes by a number of fungi including mesophilic and thermophilic strains. But the thermophilic strains are more potent due to their thermophilic nature of cellulosic proteins, which can tolerate under highly stressed conditions. Cellulase production is directly proportional to the crystallinity of biomass from which it is produced i.e., higher the crystallinity, better will be the yield of cellulases. Pretreatment of solid substrate with different chemicals act as scouring, sequestering and bleaching agent to enhance the crystallinity. It enhances the production rate of cellulases as it breaks lignin and carbohydrate bonds for successful degradation of cellulosic biomass by microorganisms[14].

CONCLUSIONS

Pretreatment of bagasse is an important parameter for the hyper production of cellulolytic enzymes as by this, lignin and carbohydrate bonds are broken down and the bagasse becomes easily susceptible to the microorganisms and ultimately the production rate enhances. The exploitation of this process on industrial scale can be a regular and better source of cellulases for a number of different applications.

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