

Cellulase Production from *Penicillium citrinum* using Brewer's Spent Grain and Pineapple Peels as Cheap, Alternate Substrates

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Abstract: Cellulases are major group of enzymes with wide-ranging industrial and biotechnological applications. The high cost of cellulase production is a major factor limiting its industrial applications in cellulose bioconversions, hence the need to develop low-cost production systems for this enzyme. Cellulose-rich plant biomass which may be agricultural or industrial in origin exists abundantly as organic wastes which are detrimental to the environment. This study evaluated cellulase production by *Penicillium citrinum* isolated from deteriorating orange fruits, using brewer's spent grain and pineapple peels as cheap, alternate substrates. Cellulase titres 3.82 ± 0.136 U/mL and 1.405 ± 0.151 U/mL were produced by the fungus, using pineapple peels and brewer's spent grain as substrates, respectively, under submerged fermentation. Maximum cellulase production by *P. citrinum* occurred with the use of pineapple peels as substrate, after 72 h fermentation period, with the use of pineapple peels at a concentration of 1.5% w/v and peptone as the best nitrogen source. The optima pH and temperature for the production of cellulase by the fungus was found to be 6.0 and 50 °C, respectively. Findings from this study indicated the potential use of pineapple peels as cheaper, alternative substrate for the production of cellulase thus mitigating its hazardous effect on the environment as pollutant. *P. citrinum* was able to grow and produce good levels of cellulase using solely pineapple peels as low-cost substrate, at high temperature of 50 °C, making this strain and this low-cost agro-industrial residue worthy of further investigation and potentially feasible for a wide range of biotechnological applications.

Keywords: Cellulase, Cellulose, *Penicillium citrinum*, Agro-Industrial Wastes, Brewer's Spent Grain, Pineapple Peels

Introduction

Cellulose is the most abundant biopolymer in nature constituting the major component of plant cell wall, followed by hemicelluloses and lignin (Lynd *et al.*, 2002; Han *et al.*, 2003; Cosgrove, 2005). Cellulose is a linear polysaccharide polymer with many D-glucose units linked by β -1,4-glycosidic bonds (Hikaru *et al.*, 2008). It has a crystalline structure stabilized by intramolecular and intermolecular hydrogen bonds (Hikaru *et al.*, 2008). Cellulose-rich plant biomass exists abundantly as organic wastes which are detrimental to the environment. This abundant biomass, which may be agricultural, urban or industrial in origin, could be utilized more efficiently by being converted to fermentable sugars which act as carbon source for the production of various products of commercial interests such as fuels and chemicals (Chen *et al.*, 2007). The natural degradation of cellulose is carried out by cellulolytic microorganisms, which form an important part of the

biosphere carbon cycle. Complete hydrolysis of cellulose to glucose is carried out by the synergistic action of three cellulolytic enzyme system (Bakare *et al.*, 2005; Eveleigh *et al.*, 2005; Gautam *et al.*, 2010). β -1,4-endoglucanase [EC3.2.1.4] randomly attacks the internal glycosidic bonds, resulting in glucan chains of different lengths; the β -1,4-exoglucanase [EC3.2.1.91] acts on the ends of the cellulose chain and releases β -cellobiose as the end products while the β -glucosidases acts specifically on the β -cellobiose by hydrolysis giving rise to two glucose molecules (Singh, 1999; Sarao *et al.*, 2010; Akinyele *et al.*, 2013). Cellulases have numerous applications and biotechnological potentials for various industries including chemicals, fuels, food, brewery, animal feed, textile, pulp and paper industries (Bhat, 2000; Ogel *et al.*, 2001; Abo-State *et al.*, 2010).

The cost of cellulase production remains a key issue in the bioconversion of cellulose-rich plant biomass

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for the production of cellulosic ethanol (Fang *et al.*, 2009; Brijwani *et al.*, 2010). Significant efforts are thus required towards lowering the contribution of enzymes to biofuel production costs (Blanch *et al.*, 2011). Organic wastes from agricultural and industrial residues, which otherwise constitute environmental pollution, are renewable sources of substrates which can be utilized for cellulase production by microorganisms. Various agricultural wastes such as wheat straw, soybean hull, corn stover, wheat bran, dairy manure and bagasse have been used for the production of enzymes (Brijwani *et al.*, 2010).

Brewer's spent grain (BSG) is a by-product of the brewery industry, being the barley malt residue obtained after wort elaboration. It consists primarily of grain husk and other residual compounds not converted to fermentable sugars by the mashing process (Xiros *et al.*, 2008). The cellulose content ranges between 9% and 25% per dry matter. The chemical composition of BSG varies according to barley variety, harvest time, malting and mashing conditions, and quality and type of adjuncts added in the brewing process. Although part of it is used as animal feed, it is still largely available, being an interesting source as raw material for the production of variety of products including lactic acid (Mussato *et al.*, 2008), breads (Stojceska and Ainsworth, 2008), and enzymes such as cellulases and hemicellulases for bioethanol production (Mussato *et al.*, 2008).

Pineapple peels is a by-product of the pineapple juice-processing industry. Its production in large quantities results in serious waste disposal problem which can lead to environmental pollution, if not utilized (Rani and Nand, 2004). Finding use, especially on a large scale, for this abundant waste material would therefore be profitable from both an environmental and economic point of view. The peel is rich in cellulose, hemicelluloses and other carbohydrates. It has a substrate potential generation of methane by anaerobic digestion and currently has limited use as animal, poultry and fish feeds (Rani and Nand, 2004; Paengkoum *et al.*, 2013).

In this study, cellulases were produced by a strain of *P. citrinum* isolated from deteriorating orange fruits using brewer's spent grain and pineapple peels as substrates. The influence of several cultural factors such as carbon sources (substrate), nitrogen sources, fermentation period, pH and temperature, on cellulase production, was then investigated.

Materials and methods

Isolation of fungi

Orange fruits, at different stages of decay, were collected from the fruits market in Ile-Ife, Nigeria, using sealed cellophane bags and transported to the laboratory of the Department of Microbiology, Obafemi Awolowo University, Ile-Ife where fungal isolations were carried out immediately. A sterilized scalpel was used to cut deeply into decayed portions of the orange fruits and sterile inoculating needle was used to collect samples which were placed on the surface of freshly prepared and sterilized malt extract agar in Petri dishes. The culture plates were then incubated at 26 °C for 4-6 days. Morphologically distinct fungal growths were subcultured onto fresh plates of malt extract agar until pure isolates were obtained. Pure fungi were then maintained on potato dextrose agar (PDA) slants at 4°C inside the refrigerator.

Characterization and identification of fungi

The fungal isolates were characterized and identified by observing their macroscopic morphological features such as surface colour on plates, texture, appearance, bottom of plates colour and diameter of colonies. The microscopic morphological characteristics observed were the spore type and shape, type of sporangia and type of hyphae. Smears of the isolated fungi were prepared with dissecting needle in lactophenol cotton blue stain on a glass slide, covered with cover slip and examined with x 40 objective of a compound binocular microscope (Barnett and Hunter, 1972; Domsch *et al.*, 1986; Benson, 1990; Lieckfield *et al.*, 1999). Four fungal isolates - *Aspergillus malignus*, *Aspergillus terreus*, *Fusarium solani* and *Penicillium citrinum* were isolated from deteriorating orange fruits collected from the fruits market in Ile-Ife, Nigeria.

Screening of fungi for cellulase production

The four fungal isolates were screened for their cellulolytic activities on carboxymethyl cellulose, as sole carbon source, under submerged fermentation condition.

Preparation of agro-industrial wastes

The agro-industrial wastes brewer's spent grain (*Hordeum vulgare*) and pineapple peel (*Ananas comosus*) were obtained locally from their dumpsites. The residues were prepared by exhaustive washing with distilled water, followed by drying at 70 °C in an oven for 2 h. They were then milled, sieved at 40 mm mesh size and stored in airtight transparent plastic containers to keep them moisture-free (Hafiz *et al.*, 2010). The substrates were treated to autoclaving for 1 h with 5% ^{w/v} NaOH (20 mL per gram of substrate) in flasks for delignification. They were then washed thoroughly with distilled water and neutralized with 1

M HCl. They were finally washed with distilled water and dried (Ali *et al.*, 1991).

Submerged fermentation and extraction of enzyme

Medium composition used for submerged fermentation (in g/L), according to the modified method of Mandels and Weber (1969), were K₂HPO₄, 0.5; KH₂PO₄, 2.0; KNO₃, 9.9; MgSO₄.7H₂O, 0.1; FeSO₄.7H₂O, 0.1; MnSO₄.7H₂O, 0.01; ZnSO₄.7H₂O, 0.01, supplemented with brewer's spent grain (5.0 g/L) or pineapple peels (5.0 g/L). Fermentations were performed in 250 mL Erlenmeyer flasks containing 100 mL of the culture medium. Standardized spore suspensions of *P. citrinum* (1 x 10⁶ spores/mL) were used to inoculate 100 mL of pre-sterilized basal medium. The fermentation culture was incubated at 35°C, under agitation on orbital shaker (180 rpm), for 5 days. After incubation, the whole content of the shake flasks were centrifuged at 5000 rpm for 10 min at 4°C using the refrigerated ultracentrifuge. This was then filtered through glass microfiber filter (Whatman GF/A, UK) and the crude supernatant used for enzymatic assays. Experiments were carried out in duplicates and results were expressed as average values.

Cellulase assay

Carboxymethyl cellulase activity was determined by measuring the release of reducing sugar glucose in a reaction mixture of 1.0 ml of the crude supernatant and 1.0 ml of 2% (w/v) carboxymethyl cellulose (CMC) sodium salt (Sigma, St Louis, MO, USA) solution in 0.05 M citrate phosphate buffer (pH 4.5) incubated at 50°C for 30 min. The control sample contained the same amount of substrate and 1.0 ml of the enzyme solution heated at 100°C for 15 min, incubated at same conditions. At the end of incubation period, the reaction was terminated by addition of 3.0 ml of 3, 5-dinitrosalicylic acid (DNSA) reagent (Miller, 1959). Each tube was incubated for 5 min in a boiling water bath and then cooled rapidly. The cellulose activity of the reaction mixture was measured against a blank sample at wavelength of 540 nm. The concentration of glucose released by enzyme was determined against a standard calibration curve of glucose concentration. One unit (U) of carboxymethyl cellulase activity corresponded to 1.0 µmol of glucose equivalent released per milliliter per min under the assay conditions according to Ghose (1987).

Screening of agro-industrial residue as substrate for cellulase production

Brewer's spent grain (BSG) and pineapple peels (PP) were each incorporated into the basal medium as sole carbon source in a concentration of 5.0 g/L with CMC serving as control. Equal 5.0 g/L concentration

combinations of CMC and BSG, and CMC and PP were also set up in flasks of basal medium. After inoculation with standardized spore suspension of *P. citrinum*, the flasks were incubated at 30 °C for 4 days at 120 rpm on a rotary shaker (Gautam *et al.*, 2010). At the end of incubation, the culture supernatant was assayed for enzyme activity using the method stated above.

Effect of varying substrate concentrations on cellulase production

The effect of varying concentrations (0.5 to 3.0 % w/v) of selected substrate (pineapple peels) on cellulase production by *P. citrinum* was determined. After inoculation with standardized spore suspensions of the isolate, the flasks were incubated at 30 °C for 4 days at 120 rpm on a rotary shaker (Gautam *et al.*, 2010). At the end of incubation, the culture supernatants were assayed for enzyme activities using the method stated above.

Effect of nitrogen sources on cellulase production

The effect of various nitrogen sources, at 5 g/L, on cellulase production was determined by supplementing the fermentative medium with various nitrogen sources (ammonium sulphate, glycine, peptone, urea, yeast extract). Fermentation was carried out at 35°C for 3 days in an orbital shaker incubator at 120 rpm. The cell-free supernatant obtained was assayed for cellulase activity and protein concentration as stated above.

Effect of pH and temperature on cellulase production

The effect of initial pH on cellulase production was determined by varying the pH values of the fermentation medium from 3.5 to 6.5. The influence of temperature on cellulase production was also determined by varying the temperature of incubation from 30 °C to 60 °C. Submerged fermentation was carried out for 3 days at 120 rpm in an orbital shaker incubator. The cell-free supernatant obtained was assayed for cellulase activity as stated above.

Effect of fermentation period on cellulase production

The influence of fermentation periods, up to 120 h, using optimal conditions established previously, was studied. The cellulase activities in fermentation media supplemented with pineapple peels as substrates were measured at 24 h intervals throughout the 120 h incubation period (Milala *et al.*, 2005).

Results

Characterization and identification of fungal isolates

Cultural, morphological and microscopical observations made on all the isolates showed that

they were all fungi. Four morphologically-distinct fungal strains were obtained from the decayed orange fruits. Based on the phenotypic characterization of the isolates such as distinctive characters of the arrangement, shapes and sizes of macro- and microconidia, presence and absence of chlamydospores as well as colony appearances and pigmentations on potato dextrose agar medium, they were identified as *Aspergillus malignus*, *Aspergillus terreus*, *Fusarium solani* and *Penicillium citrinum*.

Screening of fungal isolates for cellulase production

The four fungal isolates - *Aspergillus malignus*, *Aspergillus terreus*, *Fusarium solani* and *Penicillium citrinum* screened for their ability for cellulase production on CMC as sole carbon source. *P. citrinum* exhibited the highest cellulase activity (1.008 ± 0.079 U/mL) and was therefore selected for subsequent studies (Figure 1).

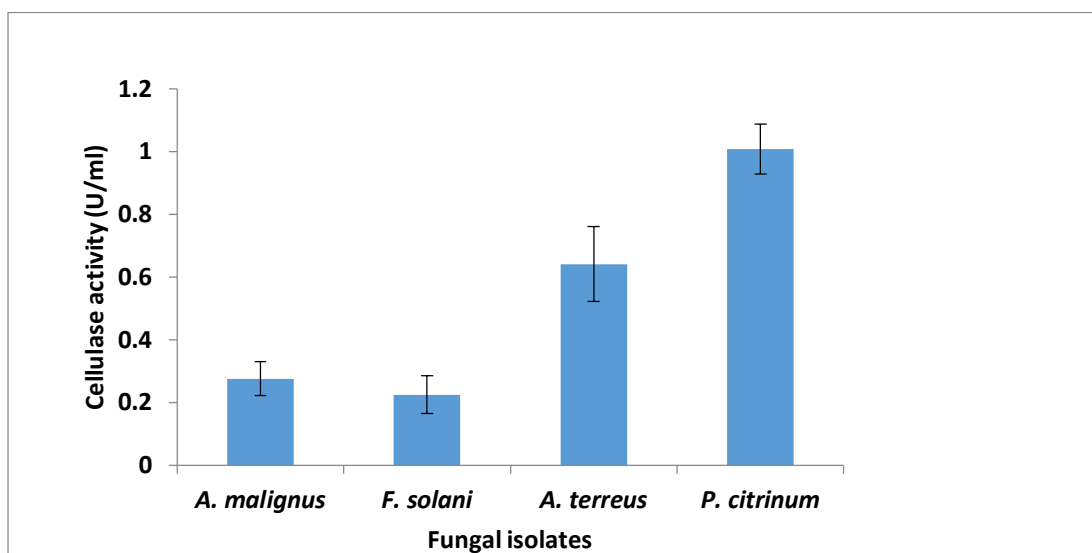


Fig 1. Screening of fungal isolates for cellulase production on carboxymethyl cellulose (CMC).

Effect of agro-industrial wastes, as substrates for cellulase production

P. citrinum utilized both BSG and PP as substrates for cellulase production but the maximum cellulase activity (3.832 ± 0.136 U/mL) was observed with the use 5.0 g/L pineapple peel as the sole carbon source

(Figure 2). The use of BSG as carbon source for cellulase production from the fungus produced enzyme activity of 1.405 ± 0.151 U/mL. The use of PP as substrate produce activity of 2.89 ± 0.173 U/mL while combinations of CMC and BSG gave the enzyme activity of 1.188 ± 0.074 U/mL (Figure 2).

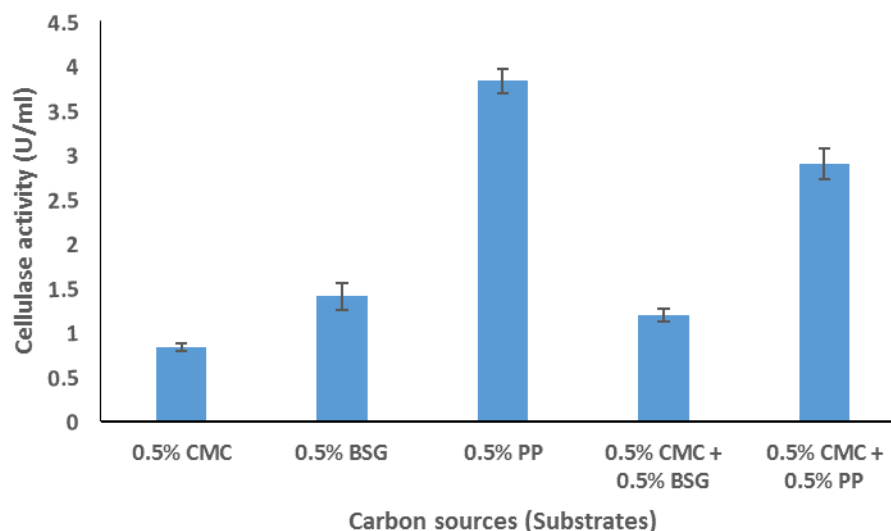


Fig. 2 Effect of different carbon sources on cellulase production by *P. citrinum*. CMC - carboxymethyl cellulose; BSG - brewer's spent grain; PP - pineapple peels.

Effect of substrate (pineapple peels) concentrations on cellulase production

Cellulase activity was studied by varying the concentrations of substrate/ pineapple peels incorporated into fermentation medium. Cellulase activity increased with pineapple peel concentration

until it reached the maximum with the use of 1.5 %^{w/v} pineapple peel in the fermentation medium (Figure 3). Substrate concentrations beyond this led to gradual decrease in cellulase activity and hence production.

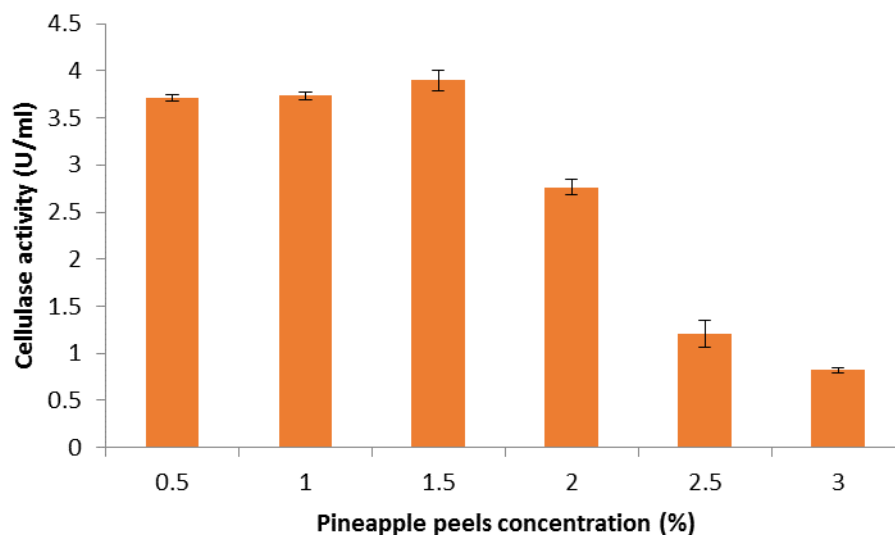


Fig. 3 Effect of varying concentrations of pineapple peels on cellulase production by *P. citrinum*.

Effect of nitrogen sources on cellulase production

The effect of nitrogen sources on cellulase production from *P. citrinum* is presented in Figure 4. Among the nitrogen sources used, peptone produced the maximum enzyme production (3.148 ± 0.136 U/mL)

followed by the use of yeast extract (2.084 ± 0.187 U/mL) and urea (2.134 ± 0.127 U/mL) while the use of ammonium sulphate produced the least enzyme activity and hence production (1.723 ± 0.052 U/mL) (Fig. 4).

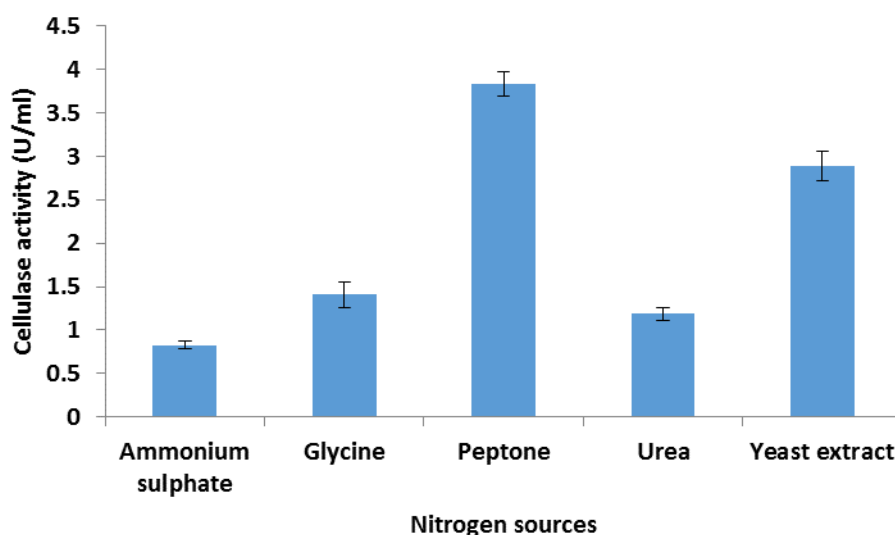


Fig. 4 Effect of different nitrogen sources on cellulase production from *P. citrinum* using pineapple peels as sole carbon source.

Effect of pH and temperature on cellulase production

The influence of pH on cellulase production from *P. citrinum* is presented in Figure 5. Cellulase activities increased with increasing pH until it reached maximum at the pH value of 6.0 (3.296 ± 0.068 U/mL). pH values above this resulted into steady decrease in cellulase activities.

The influence of temperature on the production of cellulase from *P. citrinum*, using pineapple peels as substrate, is presented in Figure 6. Maximum cellulase production was observed at 50 °C (3.202 ± 0.122 U/mL). Above this temperature, cellulase production decreased steadily (Figure 6).

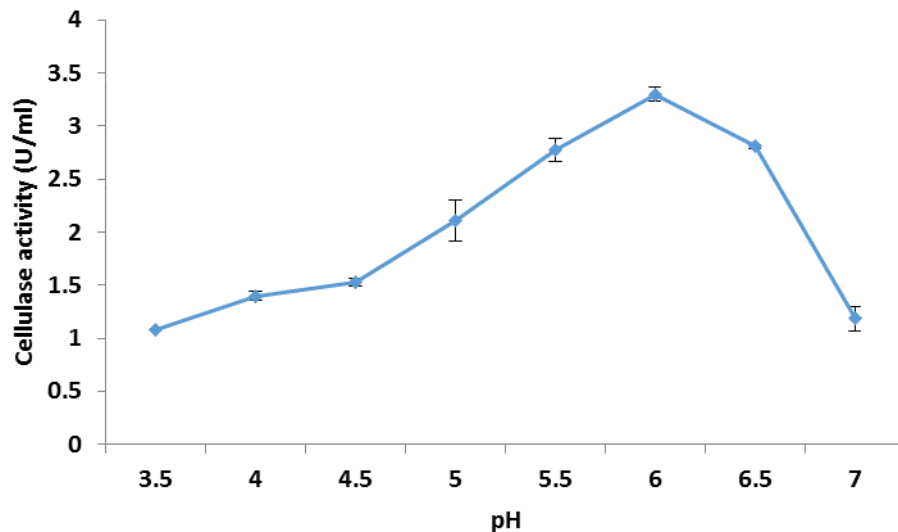


Fig. 5 Effect of pH on cellulase production by *P. citrinum* using pineapple peels as sole carbon source.

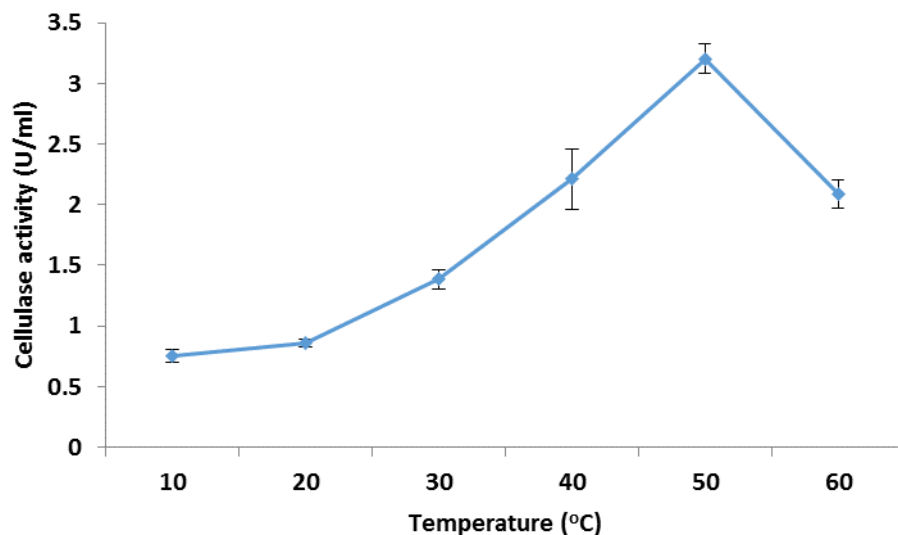


Fig. 6 Effect of temperature on cellulase production by *P. citrinum* pineapple peels as sole carbon source.

Effect of fermentation period on cellulase production

Fermentation time course profile showed considerable variation in cellulase production at different fermentation period. The cellulase

production increased with fermentation period until it reached the maximum (3.190 ± 0.144 U/mL) after 72 h incubation. Beyond this period, there was a steady decline in cellulase production (Figure 7).

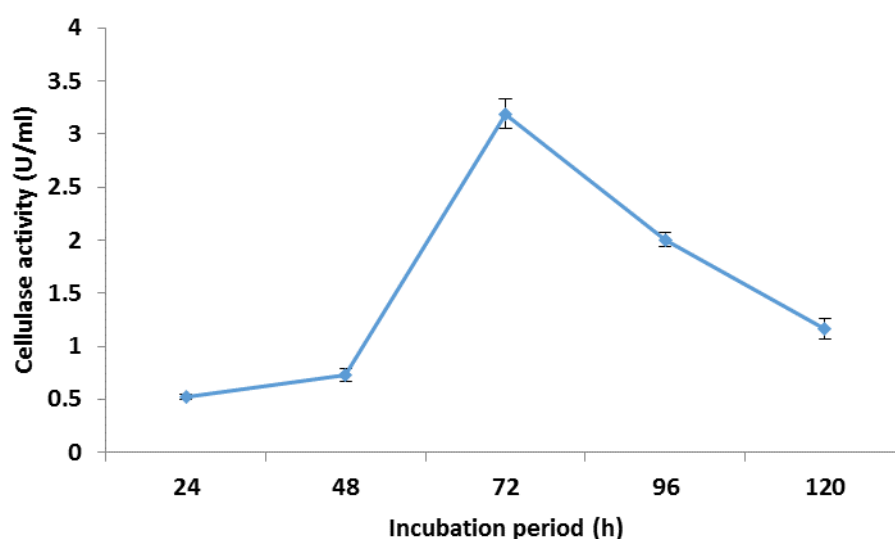


Fig. 7 Effect of incubation period on cellulase production from *P. citrinum* using pineapple peels as sole carbon source.

Discussion

In the present study, *Penicillium citrinum* has been used to produce cellulase using low-cost, readily available agro-industrial residues such as brewer's spent grain and pineapple peels, which otherwise, mostly constituted pollution in the environment. Cellulases have wide applications with potentials for use in the food processing, detergent and textile industries as well as in the conversion of agricultural (lignocellulosic) biomass into products with commercial value (Bon and Ferrara, 2007; Asgher *et al.*, 2013; Iqbal *et al.*, 2013; Irshad *et al.*, 2013). With the increasing demand for alternative liquid fuels worldwide, cellulase is also used as the primary enzyme for enzymatic hydrolysis of lignocellulosic biomass in bioethanol production process.

The production economics of bioethanol is largely dependent on the cost of cellulase. The high cost of cellulase presents a significant barrier to their use for these wide-ranging commercial applications hence economic ways of producing the enzyme has attracted worldwide attention (Fang *et al.*, 2009; Brijwani *et al.*, 2010; Klein-Marcuschamer *et al.*, 2011). For enzyme production cost to be minimized, there has to be consistent and considerable progress made in strain development, optimization of culture conditions, mode of fermentation and modelling the process (Lee *et al.*, 2005; Acharya and Chaudhury, 2012; Riswan *et al.*, 2012).

Four fungal species, isolated from deteriorating orange fruits, were screened for their ability for cellulase production, on carboxymethyl cellulose, under submerged fermentation. All the isolates

secreted cellulase into the medium with *P. citrinum* producing the highest concentration of cellulase. It was therefore selected for further studies. The difference in the amount of cellulase production from the different fungi may be attributed to differences in their genetic compositions (Gautam *et al.*, 2010).

The capacity for the hydrolytic breakdown of lignocellulosic materials is distributed among certain species of bacteria, fungi and actinomycetes (Hafiz *et al.*, 2010). Microorganisms are capable of utilizing the organic matter in wastes both as a source of energy for growth and as carbon source for the synthesis of cell biomass and essential molecules such as enzymes (Kim and Dale, 2004). Cellulolytic activities were detected to different levels when *P. citrinum* was grown in fermentation media containing different agro-industrial wastes as substrates. Among the lignocellulosic wastes tested, pineapple peels (PP) was found to be the best substrate for cellulase production followed by brewer's spent grain (BSG), equal combination of CMC and PP and equal combination of CMC and BSG. Differences in the production of cellulolytic enzymes on a variety of lignocellulosic substrates by microorganisms could be due to factors such as variability in cellulose content and nature, substrate accessibility, presence of activators and inhibitors and natural cellulolytic abilities of the organism at different conditions (Narasimha *et al.*, 2006; Mabrouk and Ahwany, 2008). Several lignocellulosic wastes have been utilized as substrates for cellulase production by several researchers. These included sawdust (Narasimha *et al.*, 2009), wheat bran (Chandra *et al.*, 2007), brewer's spent grain (Nascimento *et al.*,

2009), rice bran (Reddy *et al.*, 2015), pineapple pulp (Omojasola *et al.*, 2008) and wheat straw (Azzaz *et al.*, 2012). The use of lignocellulosic biomass for cellulase production has become a common trend in recent years since this significantly reduces costs (Adsul *et al.*, 2004; Nascimento *et al.*, 2009; Balaraju *et al.*, 2010; Ravindran *et al.*, 2010). Deposition or release of these wastes is one of the main causes of environmental pollution with the attendant health hazards. Microbial conversion into useful products such as enzymes may therefore serve to control the problems of environmental pollution. Agro-industrial wastes could be considered as promising low-cost raw materials as a result of their great availability in nature and renewable characteristics (Rubin, 2008).

A substrate concentration of 1.5%^{w/v} (15.0 g/L) pineapple peel gave the highest yield of cellulase in the fermentation medium. Further increase in concentration led to sharp decline in cellulase yield. A 1.0% substrate concentration gave the highest yield of cellulase from *Trichoderma longibrachiatum* on pineapple pulp (Omojasola *et al.*, 2008). Substrate concentration is an important factor in enzyme yield and substrate hydrolysis rate (Reddy *et al.*, 2015). Increase in substrate concentration beyond the optimum can lead to saturation which substantially lowers enzyme formation (Liu and Yang, 2007).

Maximum cellulase production was observed after 72 h fermentation period, using pineapple peels as substrate. Beyond this period, enzyme production decreased drastically. The reduction in enzyme yield might be as a result of denaturation of enzyme resulting from variation in pH during fermentation according to Krishna (1999), or due to cumulative effect of cellobiose which is known to inhibit both endoglucanase and β -glucosidase (Howell and Mangat, 1978). Our results are in consistency with those reported by Chandra *et al.* (2007), in which cellulase showed maximum activity after 72 h fermentation by *A. niger* on groundnut fodder, wheat bran and rice bran. Milala *et al.* (2005) also reported maximum cellulase activity after 72 h fermentation by *A. niger* grown on maize straw and rice husk. On the other hand, Nipa *et al.* (2006) and Akinyele *et al.* (2013) reported 96 h as the optimum fermentation period for cellulase production from *A. humicola* and *A. niger*, respectively. However, a short fermentation period for enzyme production offers potential for inexpensive production of enzyme (Sonjoy *et al.*, 1995).

Optimum pH for cellulase production was found to be 6.0. As the pH level increased, enzyme production decreased. The initial pH of the medium has a great effect on microbial growth, permeability of membrane as well as on the biosynthesis and stability of the enzyme (Shoichi *et al.*, 1985; Odeniyi *et al.*,

2009). The pH change observed during growth of microbes also affects product stability in the medium (Gupta *et al.*, 2008). A similar observation was made for cellulase production from *A. terreus* Ali *et al.* (1991) and Azzaz *et al.* (2012). However, the best pH for cellulase production from different strains of *A. niger* were observed to be 5.0 according to Pham *et al.* (2010) and 5.5 according to Akinyele *et al.* (2013).

In this study, the optimum temperature for cellulase production from *P. citrinum* was found to be 50 °C. Maximum cellulase production was observed at 40 °C for *A. terreus* according to Ali *et al.* (1991) and 37°C for *P. fellutanum* according to Kathiresan and Manivannan (2006). Temperature of incubation plays important role in the metabolic activities of microorganisms and optimum temperature for cellulase production depends on strain variation of the microorganisms (Gautam *et al.*, 2010).

Among the five nitrogen sources tested, peptone was found to be the best for producing cellulase from *P. citrinum*. Differences in cellulase production from the use of various nitrogen sources may be due to the fact that peptone may be readily available to the fungus for metabolism during growth and enzyme production as opposed to the others. Our result is in line with those of other workers (Devanathan *et al.*, 2007; Mrudala and Murugammal, 2011). Organic nitrogen sources such as peptone were also found to be more suitable for optimizing cellulase production from *Bacillus alkalophilus* S39 and *B. amyloliquefaciens* C28 than the inorganic sources (Abou-Taleb *et al.*, 2009). However, Xavier and Lonsane 1994) indicated that the source of nitrogen should be inorganic for better results. Differences in enzyme yields in different studies could be due to differences in cultural practices and different strains of organisms used (Chandra *et al.*, 2007).

Conclusions

This study has revealed that *Penicillium citrinum*, isolated from deteriorating orange fruits, exhibited unique characteristics of thermostability and the ability to utilize pineapple peels as cheap substrate for the production of cellulase. The result obtained also indicated a biotechnological control of environmental pollution through microbial bioconversion of agro-industrial wastes into commercially valuable cellulases, with wide-ranging industrial applications. Optimal cultural factors for cellulase production from the fungus were a fermentation period of 72 h, 1.5%^{w/v} pineapple peels concentration, optima pH and temperature of 6.0 and 50 °C, respectively and 0.5%^{w/v} peptone concentration as nitrogen source.

Conflict of interest

The authors declare no conflict of interest.

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