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Optimization of Conditions for Maximal Production of Recombinant Thermostable Cellulase from *Thermotoga naphthophila* using *E. coli* BL21-CodonPlus (DE3) as Expression Host

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Authors' Contribution
AK performed experiments. MT planned and supervised the study and provided guidance for manuscript write-up. ASH and TY facilitated the conduction of experiments. ARA and MW helped in data analysis. SS, SF and ARS helped in manuscript write-up.

Key words
Recombinant cellulase, BL21-CodonPlus (DE3), *Thermotoga naphthophila*, Optimization of conditions.

ABSTRACT

Current study was designed for the development of an economic and environment friendly mechanism for the production of thermostable cellulase. Production of cellulase was focused due to its diverse range of application in industry. In the present study, conditions were optimized for the maximal production of recombinant thermostable cellulase from *Thermotoga naphthophila* using BL21-CodonPlus (DE3) cells as expression host and pET28a as expression vector. Effect of various concentration of Isopropyl β-D-1-thiogalactopyranoside (IPTG), post induction time, effect of temperature and pH were examined for the maximal production of recombinant cellulase. The effect of supplementation of LB medium with additional carbon and nitrogen sources was also analyzed for maximal production of recombinant protein. Higher level enzyme activity was recorded at 25°C, pH 7.0 when the cells were induced with 0.5 m*M* IPTG with 22h post induction incubation. Supplementation of LB medium with 1% glucose and yeast extract enhanced the production of recombinant thermostable cellulase. Enzyme showed strong potential for its use in paper and poultry feed industry. Under the optimal conditions we could able to produce 48 U/mL of recombinant cellulase.

INTRODUCTION

Cellulose is the most abundant organic compound on earth and is the principal component of plant cell wall (Wang *et al*., 2015). It is a homo-polymer of glucose where monomers are linked each other through β-1,4 glycosidic linkage (Kang *et al*., 2011). A variety of industrial and agricultural wastes have cellulose as a major component which can be transformed into simple sugar via the hydrolysis process. Two methods are available for hydrolysis of cellulose to its monomeric units. The chemical hydrolysis method requires the treatment of cellulose with strong acid under high temperature >300°C at 25 MPa which make this method as cost efficient with poor quality and low yield of final product (Deguchi *et al*., 2006). Enzymatic hydrolysis method is preferred over chemical hydrolysis due to its environment friendly nature. Cellulases are biocatalyst required for digestion of cellulose under ordinary temperature and pH conditions which is not hazardous to environment (Koomnok, 2005).

On the basis of catalytic action, the cellulases have been divided into the three major classes including endoglucanases (EC 3.2.1.4), β-glucosidase (EC 3.2.1.21) and exoglucanase (EC 3.2.1.91) which are responsible for the de-polymerization of complex cellulose to simple glucose units (Karmakar and Ray, 2011). Cellulases have been immensely used in various industries including textile industry for improvement of fiber softness (Galante *et al*., 1998; Sreenath *et al*., 1996), in paper and pulp industry for refinement and strengthing of paper (Singh *et al*., 2007; Akhtar, 1994; Bhat, 2000), in bioethanol production for saccharification of lignocellulosic materials (Sukumaran *et al*., 2005; Kuhad *et al*., 2010; Gupta *et al*., 2011), in wine and brewery industry for improving

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the quality and yield of the fermented product (Singh *et al*., 2007; Galante *et al*., 1998; Bamforth, 2009), in food processing industry for the improvement of extraction procedure, cloud stability, texture and clarification of fruit and vegetable juices (Minussi *et al*., 2002; Carvalho *et al*., 2008), in animal feed industry for the improvement of nutritional value of feed by adding the digestion process and ultimately the performance of animals (Dhiman *et al*., 2002), in agriculture for enhancement of crop growth by controlling plant diseases (Chet *et al*., 1998; Harman and Kubicek, 1998) and in detergent industry for improving colour brightness, feel and dirt removal from garments (Sukumaran *et al*., 2005; Singh *et al*., 2007; Sharyo *et al*., 1978).

Recombinant DNA technology played significant role for the fulfilment of industrial requirement of enzymes. Selection of appropriate expression vector and its compatibility with expression system is very important for the successful production of recombinant protein. An efficient expression system which can fulfil the industrial demand of enzyme in a limited time with low cost is required in order to meet the industrial demand (Porro *et al*., 2005). Optimization of conditions is another strategy being followed by scientists for the higher level production of recombinant proteins (Larentis *et al*., 2011; Couto *et al*., 2017; Mohajeri *et al*., 2016; Morowvat *et al*., 2015; Muntari *et al*., 2012).

Present study deals with the utilization of BL21-CodonPlus (DE3) a prokaryotic expression system as expression host and pET28a having cellulase gene from *T. naphthophila* as expression vector for the production of recombinant cellulase. The conditions including inducer concentration, post induction time, medium composition, temperature and pH were analyzed for the enhanced production of recombinant cellulase.

MATERIALS AND METHODS

Chemicals

All the chemicals utilized in this study were of analytical grade and were purchased from Merck, Life Sciences, Darmstadt, Germany.

Expression machinery

Recently, we have characterized a recombinant thermostable cellulase from *T. naphthophila* (Khalid *et al*., 2019). Cellulase gene from *T. naphthophila* was cloned in pET28a and was expressed in BL21-CodonPlus (DE3) cells. The recombinant cells harboring the pET28a with cellulase gene from *T. naphthophila* were utilized for the optimizing of conditions for the maximal production of recombinant thermostable cellulase.

Production of recombinant cellulase

Regarding the production of recombinant cellulase, the overnight grown recombinant BL21-CodonPlus (DE3) cells were diluted to 1% with fresh LB medium and was incubated at 37°C under shaking conditions (I3000, Lab Tech, Korea) till the OD_{660} reached to 0.4. The cells were induced with 1 m*M* IPTG followed by further incubation at 37°C. The production of recombinant protein was analyzed after lyzing the cells by sonication (Sonics, Newtorn, USA) (Mansoor *et al*., 2018).

Activity assay

Cellulase activity was determined in 50 m*M* sodium acetate buffer (pH 4.8) using carboxymethyl cellulose as substrate. The reaction mixture was incubated in water bath at 90°C for 30 min. The production of monomeric sugars was estimated by DNS method. One unit of enzyme was the amount of enzyme required to liberate 1 µmol of reducing sugars under the assay conditions (Miller, 1959).

Optimization of conditions for the maximal cellulase production

Effect of IPTG concentration

In order to explore the effect of inducer on the production of recombinant protein, the BL21-CodonPlus (DE3) cells were induced by varying the IPTG concentration from 0.1 to 1 m*M* and cellulase production was recorded at each IPTG concentration (Pereira *et al*., 2010).

Effect of post-induction temperature and incubation time

The production of recombinant cellulase was analyzed at 20, 25 and 37°C. For this purpose, the cells were shifted to respective temperature before induction after attaining the OD_{660} to 0.4 and were induced with 0.5 m*M* IPTG. The cellulase production was recorded at 20, 25 and 37°C. Regarding the optimization of incubation time, the sample after induction was withdrawn after every hour and was utilized for the determination of enzyme activity (Oelschlagel *et al*., 2015).

Effect of pH

The overnight grown recombinant BL21-CodonPlus (DE3) cells were diluted to 1% with fresh LB broth prepared in 50 m*M* of each of sodium acetate buffer (3-5), Sodium phosphate buffer (5-7) and Tris HCl buffer (7-9). The cells were incubated again at 37°C till the achievement of OD_{660} to 0.4 and were induced with 0.5 m*M* IPTG followed by further incubation at 25°C for 22 h. The enzyme activity was recorded at each pH after losing the cells (Oelschlagel *et al*., 2015).

Effect of supplementation of medium

Various carbon sources including glucose, sucrose, lactose, starch, fructose & maltose and nitrogen sources including yeast extract, tryptone, peptone, urea, glycine and inorganic nitrogen sources including ammonium chloride and ammonium sulphate were utilized for the supplementation of LB medium at a final concentration of 1%. The overnight grown recombinant BL21-CodonPlus (DE3) cells having recombinant pET28a were diluted to 1% with the LB medium supplemented with additional carbon and nitrogen sources separately. The cells were induced with 0.5 mM IPTG and the expression of recombinant protein was analyzed at 25°C after 22 h of incubation (Zhang *et al*., 2009).

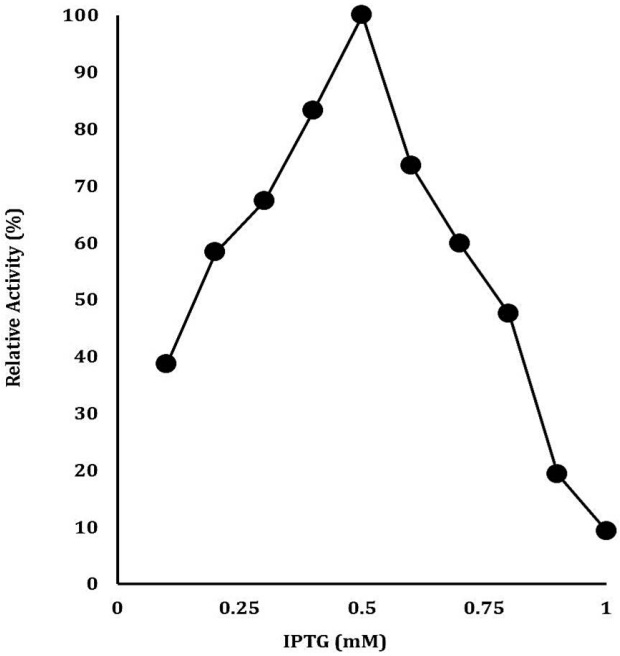

Fig. 1. Optimization of IPTG concentration for the maximal production of recombinant cellulase. The data on X-axis shows the IPTG concentration (m*M*) whereas on Y-axis shows the relative activity (%).

RESULTS AND DISCUSSIONS

Prokaryotic expression system is being preferred over eukaryotic expression system due to its low cost, rapid growth rate, flexibility and ease to scale up for higher level production of proteins which don't require post translational modifications (Porowinska *et al*., 2013). More over optimization of conditions is another strategy along with the suitable expression system for eminent production of recombinant proteins. IPTG is an inducer molecule, commonly utilize for production of recombinant protein being produced under the lac operon. Current study demonstrated the enhanced production of recombinant protein with the increase in IPTG concentration. Maximal cellulase production was achieved at 0.5 mM IPTG while further increase in IPTG resulted in reduced enzyme production (Fig. 1). The reduced production of recombinant cellulase at higher concentration of IPTG is due to its toxicity to cell and its ability to diminish the rate of synthesis of ribosomal RNA (Rizkia *et al*., 2015). These results are in agreement with the previous report for the production of cellulases from *E. cellulosolvens* (Yoda *et al*., 2005), *C. saccharolyticus* (Park *et al*., 2011) and *T. maritima* (Pereira *et al*., 2010) whereas in contrast to this, maximal cellulase production from *P. furiosus* (Kataoka and Ishikawa, 2014) was recorded at 0.1 m*M* IPTG.

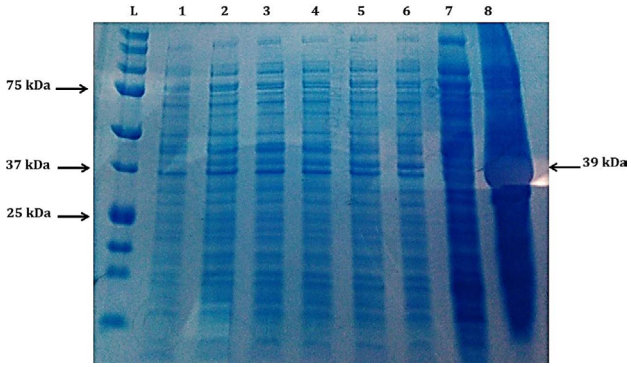

Fig. 2. Coomassie Brilliant Blue R-250 Stained SDS-PAGE Gel Showing effect of incubation temperature on the production of recombinant cellulase (39 kDa): Lane L, protein ladder (Precision Plus Protein Ladder, Bio RAD, USA); Lane 1, soluble fraction after lysis of cells having pET28a without insert as negative control; Lane 2-6, Soluble part after lysis of cells having pET28a with cellulase gene with post induction period of 1–5 h at 37°C; Lane 7, soluble part after lysis of cells having pET28a with cellulase gene with post induction period of 22 h at 20°C; Lane 8, soluble part after lysis of cells having pET28a with cellulase gene with post induction period of 22 h at 25°C.

Optimization of post induction incubation temperature is important for the higher level production of recombinant protein in soluble form. The recombinant protein was produced mainly as insoluble inclusion bodies at 37°C and the expression of recombinant cellulase was quite low at 20°C whereas the maximal soluble production of recombinant protein was achieved at 25°C (Fig. 2) after 22 h of incubation (Fig. 3). We could produce maximum of 18, 28 and 08 U/mL of recombinant cellulase at 20, 25, 37°C, respectively. This strategy of expressing protein at low temperature with extended incubation time was previously reported by Rincon *et al*. (2017), Tayyab *et al*.

(2011) and Zhang *et al*. (2009). Cellulases from *T. maritima* (Pereira *et al*., 2010), *Acidothermus cellulolyticus* (Wang *et al*., 2015), *Eubacterium cellulosolvens* (Yoda *et al*., 2005) and *Fervibacterium nodosum* (Wang *et al*., 2010) showed their optimal production under low temperature between 16 to 28°C whereas cellulase from *Caldicellulosiruptor saccharolyticus* (Park *et al*., 2011) showed its high level production at 30°C with overnight incubation.

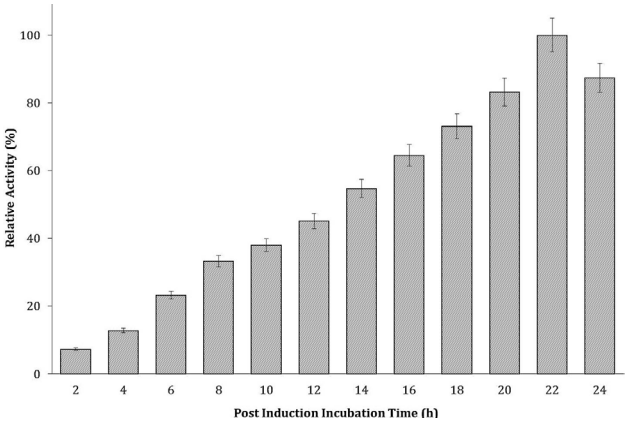

Fig. 3. Effect of post induction incubation time on recombinant cellulase production. Experiment was conducted at 25°C. The data on X-axis shows the post induction incubation time (h) and Y-axis shows the relative activity (%).

Table I.- Effect of various carbon and nitrogen sources on the production of recombinant cellulase.

Sources		Relative activity (%)
Carbon sources	Control	100
	Glucose	163
	Maltose	161
	Lactose	153
	Sucrose	127
	Fructose	109
	Starch	105
Nitrogen sources	Control	100
	Yeast extract	197
	Tryptone	171
	Peptone	120
	Ammonium sulphate	40
	Ammonium chloride	38
	Urea	34
	Glycine	23

Change in pH always shows significant influence on the growth of bacteria. No significant bacterial growth or cellulase production was recorded at pH 3 or 4. However the cellulase production was increased with the increase in pH beyond 5 and maximal cellulase production was recorded at pH 7 while further increase in pH resulted in reduced enzyme production (Fig. 4). These findings are in agreement with the findings of Oelschlagel *et al*. (2015) while according to other reports optimal production of recombinant proteins was recorded at pH 7.5 (Wang *et al*., 2014; Zhang *et al*., 2009; Mendoza *et al*., 2014).

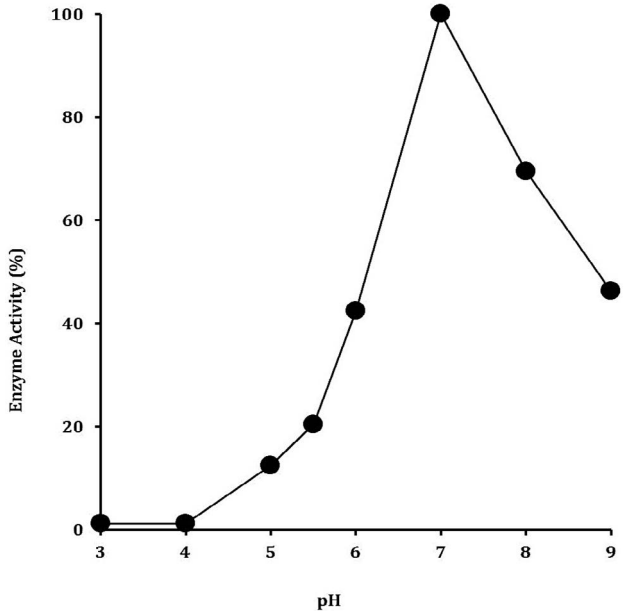

Fig. 4. Optimization of pH for the maximal production of recombinant cellulase The cellulase production was analyzed in 50 m*M* of each of sodium acetate buffer (3-5), sodium phosphate buffer (5-7) and Tris HCl buffer (7-9). The data on X-axis indicates the buffer pH values while on Y-axis show relative activity (%).

Medium supplementation with various carbon and nitrogen sources showed the enhanced production of recombinant cellulase. The high level production of recombinant protein was achieved when the medium was supplemented with glucose or maltose at a final con centration of 1%. The results depicted that glucose or maltose are carbon sources being preferred by BL21-CodonPlus (DE3) cells for their growth and for the production of recombinant protein (Table I). This is might be due to their monomeric nature and ease in absorption as compared to lactose or sucrose (disaccharides) and starch (polysaccharide). Similarly, aldo-sugars are being preferred over keto-sugars by BL21-CodonPlus (DE3) cells for their growth. These results are in agreement with previous reports by Nur *et al*. (2016), Bren *et al*. (2016), Wang *et al*. (2015) and Bettenbrock *et al*. (2007). Production of

recombinant cellulase was also enhanced when the LB medium was supplemented with yeast extract or tryptone as additional nitrogen sources whereas the rest of selected nitrogen sources could not contribute significantly in the production of recombinant protein (Table I). The results demonstrated that BL21-CodonPlus (DE3) cells preferred organic nitrogen sources over inorganic nitrogen sources for the growth (Table I). These results are in agreement with previous reports by Fu *et al*. (2006), Zhang *et al*. (2009), Mahmoudi *et al*. (2012) and Lee and Chang (1994), who observed the higher level cellular growth and the enhanced production of recombinant protein when the medium was supplemented with yeast extract and tryptone, respectively.

CONCLUSION

This study demonstrated the optimization of conditions for the enhanced production of recombinant thermostable cellulase using a prokaryotic expression system. Optimization studies revealed the highest cellulase activity when recombinant cells were induced with 0.5 mM IPTG with post induction incubation of 22h at 25°C (28 U/mL) in the presence of 50 m*M* sodium phosphate buffer pH 7 (37 U/mL) and when the LB medium was supplemented with glucose and yeast extract as carbon and nitrogen sources (48 U/mL). Current work was a foundation study that will act as milestone for the industrial scale production of recombinant cellulase at domestic level and for the fulfilment of local industrial requirement of the enzyme in Pakistan.

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Statement of conflict of interest

The authors have no conflict of interest.

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