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Optimization of Conditions for the Production of Recombinant Cellulase By using *E.coli* Bl21 Codon Plus In Fermenter

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Cellulose is the richest plant biomass on earth and is an unbranched polymer composed of D-glucose residues joined by ß-1, 4-D-glycosidic bonds. The utmost abundant carbohydrates in nature are considered to be cellulases and hemicellulases. Cellulases are inducible enzymes that catalyze the hydrolysis of a ß-1, 4-glycosidic bond to release the glucose units in a cellulose molecule. Thermophilic cellulases are relatively expensive and a very significant industrial enzyme. In this study, the recombinant plasmid pET22b (+) containing the cellulase encoding gene was transformed in E.coli BL21 codon plus. A Shake flask fermentation study was performed using modified M9NG media. Lactose and IPTG were used as an inducer. After SDS-PAGE analysis, the predicted molecular weight of a protein was 62kDA Batch culture fermentation was performed using LB and modified M9NG media. Lactose was used as the cheapest inducer. Under optimized fermentation conditions, the enzyme displayed maximum activity at 37oC and pH 7. The specific activity of the enzyme was 70U/ml. The production of the recombinant enzyme was enhanced approximately 6 times in E.coli BL21 as compared to wild type strain. The expression level of the recombinant cellulase was round about 30%-40%. Molecular cloning of the cellulase encoding genes resulted in the maximum production of the cheapest enzymes that can be used for industrial purposes.

Keywords: Cellulases; M9NG; IPTG; Heteropolymers; Glucomannan; Arabinoxylan; Galactoglucomannans.

The increment in the world economic growth results in the excessive use of energy consumption. Conventional fossil fuels can fulfill the increase in energy consumption. Due to an increase in energy demand, fuels are going to be depleted constantly. It is predicted that in 2050 global oil production will splash to nearly 5 billion barrels (Campbell and Laherrere, 1998). Due to the greenhouse gas effect, public health and the environment are also poorly affected by fossil fuels. To make environment-friendly and cleaner fuel, the most convenient and profitable form of renewable energy is now being produced by several industries and research laboratories. Plant biomass is now

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widely used. Nowadays, biotechnology products and biofuels are created by advanced metabolic engineering and synthetic biology (Keasling *et al*., 2008; Lee *et al*., 2008; Tai and Stephanopoulos, 2012). Cellulose is the most abundant plant biomass on the earth (Schwarz, 2001). Being the part of complex plant cell walls with an estimated synthesis rate of 4 × 109 tons per year (Parsiegla *et al*., 1998). Due to its recalcitrant nature, it is not readily used by many microorganisms. It is an Unbranched polymer composed of D-glucose residues joined by ß-1, 4-D-glycosidic bonds. Its molecular weight ranges from 5 × 104 to 2.5 × 106 Daltons depending upon the source. Chain length varies between 100 – 14,000 residues with intra and inter-molecular hydrogen bonding. Approximately 30 individual cellulose molecules are assembled into larger units (protofibrils), which accounts for the formation of rigid, insoluble and crystalline units which are 100 – 40,000 nm long and 2 -20 nm diameter (microfibrils). These form the structurally strong framework in the cell walls. These molecules undergo self-assembly at the site of biosynthesis (Brown *et al*., 2000). Naturally, Cellulose never exists in pure form, except in few cases like cotton balls. The matrix is composed of polymers like lignin and hemicelluloses. The fibrils are usually inserted in a matrix.

Heteropolymers consist of pentoses and hexoses e.g. xylose, arabinose, glucose, and galactose respectively. Plant biomass comprises of about 15-35% of heteropolymers. A relative quantity of methyl or acetyl substituted sugars, galacturonic acid, and Glucuronic acid are also exist in plant biomass. In a few seaweeds, a xylose residue homopolymer that is Homoxylan is present e.g. red and green algae. Different kinds of hemicelluloses usually depend upon the proportion of monomers in it e.g. arabinoxylan, glucomannan, xyloglucan, and glucuronoxylan, etc. The utmost plentiful form of hemicelluloses are Xylans that are mainly the part of secondary cell walls like hardwoods. While the other hemicellulose types i.e. glucomannans and Galactoglucomannans are found in softwoods (Gírio *et al*., 2010; Sadhu and Maiti. 2013).

Lignocellulose Hydrolyzing Enzymes

Through enzyme hydrolysis, plant biomass can be converted into reducing sugars. The utmost abundant carbohydrates in nature are considered to be cellulases and hemicellulases. Due to variety in plant cell wall, there is a lot of variation in cellulolytic microbes and the related enzymes. For the hydrolysis of ß-1, 4-linkage in cellulose, cellulase can be utilized by some entities for hydrolysis purpose and results in the release of glucose molecules. The cellulose shelf life is predicted to about millions of years beyond these enzymes (Wilson, 2011). A huge diversity of hydrolytic enzymes are required for hemicellulose deterioration. Endoxylanase, ß-xylosidase, a-glucuronidase or a few esterases are essential for xylan degradation whereas in order to break the backbone, glucomannan hydrolysis needs ß-mannanse and ßmannosidase. A huge diversity of bacteria and fungi perform function during natural degenerative system for the alteration of insoluble cellulosic substrates to soluble sugar like xylose, glucose and cellobiose (Bayer *et al*., 1998).

Cellulases are inducible enzymes that catalyzes the hydrolysis of a ß-1, 4 glycosidic bond to release the glucose units in a cellulose molecule (Nishida *et al*., 2007). The biochemical conversion of cellulose polymer is catalyzed by extra cellular cellulases enzyme system throughout degradation by microorganisms.

Cellulase Components

Three components of cellulases are engaged in cellulose degradation.

Conversion of long chains of cellulose molecule to smaller fragments is catalyzed by (an endoglucanase) ß-1, 4 glucan glucanohydrolase.

An exoglucanase (ß-1, 4 glucan cellobiohydrolase performing from the non -reducing point of the cellulose chain.

The glycosidic bond of cellobiose and cellodextrins is broken down by ß-1,4 glucosidase, to release the glucose molecules that can easily enter into the cell (Bhat and Bhat, 1997).

Types of Cellulase Systems

Commonly two kinds of cellulase systems occur: one kind comprises of extracellular cellulases that work cooperatively to break down the cellulose whereas the second one is the "cellulosome" in anaerobic bacteria like Clostridium thermocellum is an enzyme complex that performs cooperatively to break down the cellulose and hemicellulose (Mathew *et al*., 2008).

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Metabolic Engineering of Cellulase and Related Enzymes

The activity and other characteristics e.g. thermal stability of cellulolytic enzymes can be improved using site-directed mutagenesis or rational design as well as directed evolution through random mutagenesis. These techniques may be applied alone or used in concert with other approaches. Prior knowledge of three-dimensional structure and mechanism of enzyme action is required for site-directed mutagenesis. Therefore, it is used only to examine the outcome of specific amino acids on protein structure or enzymatic activities. On the other hand, random mutagenesis is similar to evolution: the amino acids are randomly mutated and the gene that encodes for the improved protein is selected. It does not require structural or mechanistic information. However, it requires an efficient screen for mutant enzymes after the expression of the mutant genes.

Fermentation Strategies for Cellulase Production

Presently, the increased demand for microbial cellulases in industries is being fulfilled by extensively studied genetically engineered strains of Hypocrea jecorina and through submerged fermentation (SmF) processes. However, SmF systems have deviated towards solid-state fermentation (SSF) because SmF systems are highly costly due to maximum fermentation time with minimum production. Submerged cultures are easy to handle and monitor (Sukumaran *et al*., 2005). The developed bioreactor design, operation regulators and applicable technology may mark it useful e.g. after concentration the enzyme in SSF crude product can be straightforwardly utilized in biotechnological applications. Solid-state fermentation suggests several benefits e.g. a smaller amount of effluent production, high yield, comparatively maximum product concentration, so it may become an economical method for the production of cellulases. When cellulase production was compared in SmF and SSF, it revealed that there were a ten times decrease in the production cost in SSF (Tengerdy, 1996).There have been efforts under the process to harvest the cellulase through fed-batch cultures which aids to disallow the repression as a result of increase in reducing sugars instead of feed batch cultures (Silva *et al*., 2005).

Applications of Cellulases

There is number of prospective applications of thermophilic cellulases in genetic engineering, textile, food, feed, fuel, chemical industries, waste management, medical industry and protoplast production (Moussa *et al*., 2000).

Food Processing

Thermophilic cellulases perform a vital role in food biotechnology. The enzyme complex called macerating enzyme complex (cellulases, xylanases and pectinases) is being utilized for removal and elucidation of vegetable and fruit juices in food industries (Bhatt, 2000 : Niehaus, 1999).

Textile Industry

Thermophilic cellulases are being used for the designing of stone washed appearance in jeans, bio shining of cotton and different cellulosic materials (Van *et al*., 2001: Vieille *et al*., 2001).To minimize the decolorization and fuzzing effects as a result of numerous washes, cellulases are also being added in the detergents (Zhou *et al*., 2001: Csiszár *et al*., 2001).

Fuel Production

The concept of biofuel was first conceived in 1970s when the world faced oil crisis. Although plant biomass is very complex in chemical composition and its use for biofuels requires several steps. Its abundant supply and renewable nature makes it a potential source (Dellomonaco *et al*., 2010). Lignocellulosic materials are also used for bioethanol production but recalcitrance is the major challenge for their use. Secondly, since lignin hinders the biological conversion of Lignocellulosic biomass by preventing the access of hydrolyzing enzymes, carbohydrate part of these materials i.e. cellulose and hemicelluloses is separated from lignin by pretreatment and then these polymers are depolymerized by hydrolyzing enzymes, cellulases and xylanases(Aristidou *et al*, 2000:Himmel *et al*., 2007).

One of the biggest hinderance for

commercialization of biomass refineries is the highest cost of cellulase. The transmission of greenhouse gases and the global crude oil dilemma triggered the manufacturing of enzymatic hydrolysis of lignocellulose material. Therefore, it is an intense need to increase the cellulase productivity and reduce the enzyme cost. The objective of the present study was to increase the production of recombinant cellulase in a bioreactor by optimizing the conditions. Under optimized conditions, we enhanced the production of cellulase enzyme in a minimum time period by using a cheap source.

MATERIALS AND METHODS

Bacterial Strain and Plasmid

The pET22b (+) expression vector consisted of cellulase encoding gene and non-transformed E.coli BL21- Codon plus cells that was utilized for the recombinant cellulase expression were provided by the University of Lahore research lab (CRIMM).

Competent cells Preparation

The protocol used for competent cell preparation is given below. An agar plate was taken and E.coli BL21-Codon plus non-transformed cells were streaked on plate, then incubated overnight at 37oC (Memmart, GmbH, INB 200 E212.0477). A single colony was picked from the overnight incubated agar plate and shifted into 10ml LB media. It was grown at 37oC overnight in shaking incubator (Robus Technologies, S1 1900R). The overnight incubated bacterial culture was shifted into an ice-cold polypropylene tube of 50ml, then placed the tube on ice for 10min. At 10,000rpm, the culture was centrifuged for 10mints. After this, the upper layer of supernatant was discarded and the tube was positioned in an inverted position on a clean tissue paper for almost 1 minute to remove the remaining drops of media. Then the pellet was resuspended in 30 ml of ice-cold 0.1M CaCl2 by gentle vortexing (Velp, Italy, ZX3). The cells were retrieved by centrifugation at 10,000rpm for 10 mints. The supernatant was removed from the pellet and the pellet was resuspended in 2ml of ice cold 0.1M CaCl2 by gentle vortexing. The competent cells were distributed into aliquots and stored at -800C (ARCTIKO, Denmark, ULUF 450) or used directly for transformation.

Transformation of E.coli BL21 Codon Plus with Cloned pET22b (+)

E.coli BL21 Codon plus competent cells were removed from -800C (ARCTIKO, Denmark, ULUF 450) and let them thawed on ice and mix gently. 25µl of E.coli BL21 Codon plus competent cells and 0.5µl of cloned pET22b (+) were taken in a 1.5ml of microfuge tube. Both were mixed for a few seconds by finger flicking tube and then placed on ice for 30 mints. The mixtures are then heat

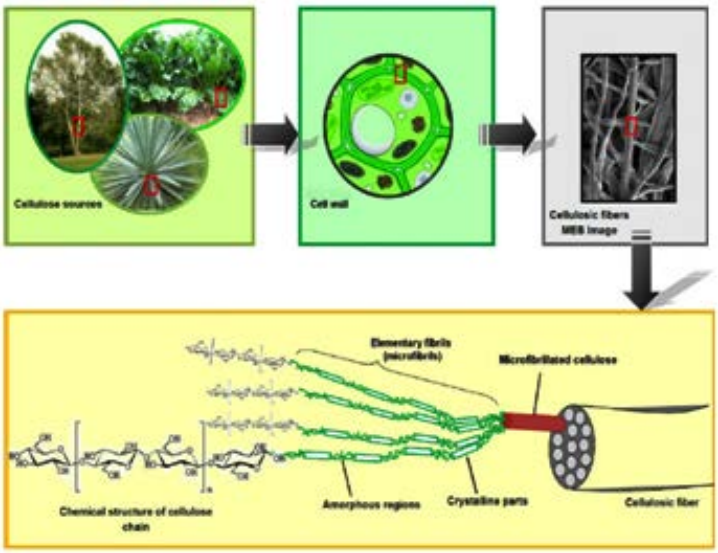

Fig. 1. Starting from cellulose sources leading to structure (Lavoine et al., 2012)

shocked by placing at 420C water bath (GmBH & CO, WNB-10) and again replaced on ice for 2-3 minutes. 900µl of SOC medium was added and incubated the tube at 200rpm for 30 mints at 370C. 50µl of transformed cells were spread on LB agar plates containing specific antibiotic (ampicillin 100µg/ml) with the help of spreader and placed in 370C incubator (Memmart, GmbH, INB 200 E212.0477) overnight and the next day placed in the 4oC.

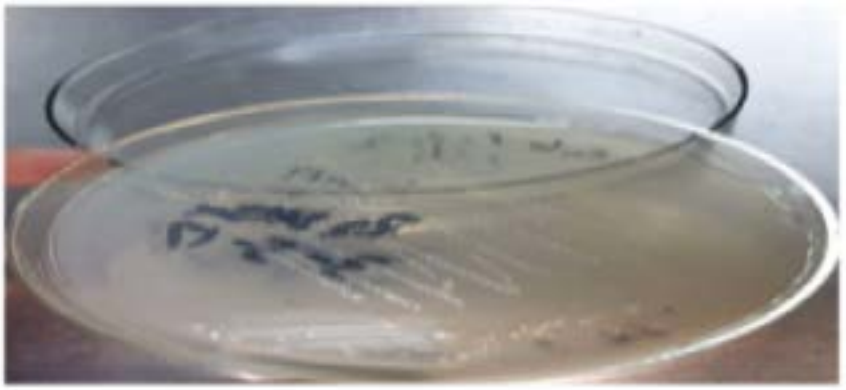

Fig. 2. Transformed *E.coli* BL21 codon plus having pET22b (+)

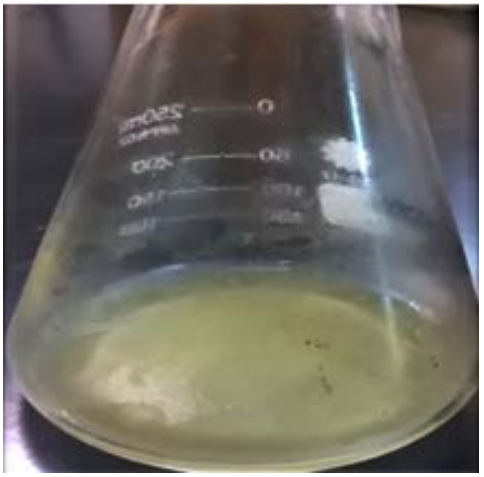

Fig. 3. LB media containing the transformed *E.coli* BL21 Codon plus cells showing the positive result of screening (PI)

Screening of Transformed Cells

For the screening of transformed cells, a single colony was selected from the overnight incubated LB agar plates and further inoculated in 10 ml of LB medium (primary inoculum) containing 100µg/ml ampicillin incubated at 370C on shaking incubator and grew overnight.

Shake Flask Fermentation Study

Determination of Optimum Temperature.

For determining the optimum temperature, the temperature was adjusted at 28ºC, 32ºC, 34ºC, 37ºC, 40ºC, and 42ºC in LB media.

Determination of Optimum pH

For determining the optimum pH, the pH was adjusted over a wide array of pH ranging from 5.0 to 6.0 in LB media.

Table 1. Determination of Optimum Temperature for Cell Growth

Time (hrs.)	Tem (°C)	$O.D_{600nm}$	Wet cell weight g/L	Expression
0	28	1.5	1.8	18%
2	32	2.6	3.1	22%
4	34	3.8	4.5	24%
6	37	6.2	7.4	30%
8	40	2.3	2.7	12%
10	42	0.5	0.6	10%

Table 2. Determination of Optimum pH for Cell Growth

Time (hrs.)	pH	$O.D_{600nm}$	Wet cell weight g/L	Expression
0	5	2.5	3	-
2	6.0	3.6	4.3	-
4	7.0	5.5	6.6	30%
6	8.0	1.2	1.4	12%
8	9.0	0.5	0.6	10%

Auto-Induction using Lactose

The shake flask fermentation study was performed as fellows. A10µl of primary inoculum was refreshed in 10ml of LB media containing 100µg/ml ampicillin at 37oC on shaking incubator and grew overnight (secondary inoculum). A 20µl of the secondary inoculum was added to the 20ml of modified M9NG medium (1%tryptone, 0.5%yeast extract, 1%NaCl) containing 100µg/ml ampicillin, 0.5%glycerol, 0.005% glucose, 0.025M K2HPO4, 0.025M NaH2PO4, 0.015M NH4Cl, O.008M MgSO4.7H2O, 1mM trace metals and auto-induced the cells with 10mM lactose and incubated at 370C, 200rpm on shaking incubator (Robus Technologies, S1 1900R) overnight. The cells were harvested by centrifuge at 10,000rpm. The protein expression was analyzed using SDS-PAGE.

Time Based Induction using IPTG

In alongside experiment, 20ml of modified M9NG medium (1%tryptone, 0.5%yeast extract, 1%NaCl) containing 100 µg/ml ampicillin, 0.5% glycerol, 0.025% glucose, 0.025M K2HPO4, 0.025M NaH2PO4, 0.015M NH4Cl, 0.008M MgSO4.7H2O, 1mM trace metals was inoculated with 20µl of secondary inoculum and placed at 370C on shaking incubator (Robus Technologies, S1 1900R).When the OD600nm reached 0.6-0.7, the cells were induced with 0.5mM IPTG and again placed at 370C shaking incubator for four hours.

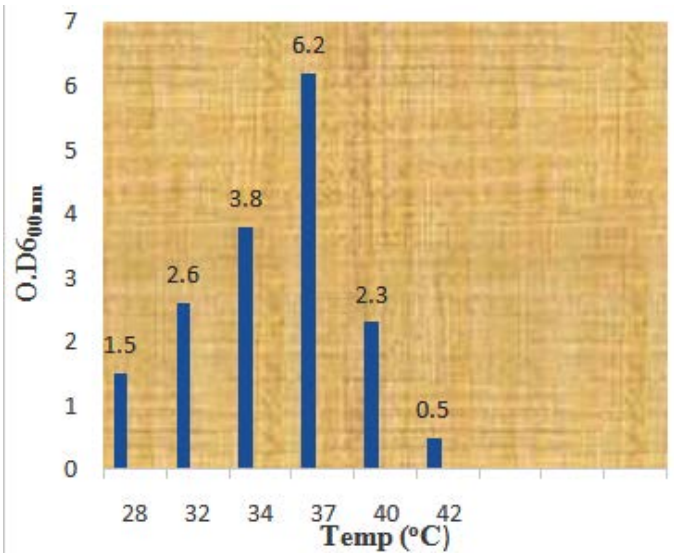

Fig. 4. Effect of Temperature on cell growth.

Table 3. Time based production of cellulase in LB media using batch fermentation

Time (hrs.)	$O.D_{600nm}$	Wet cell weight g/L	Expression.
0	0	0	-
2	0.23	0.27	-
4	0.65	0.78	-
6	1.8	2.16	8%
8	4.6	5.52	12.5%
10	8.4	10.0	18.6%
12	11.5	13.8	23.9%
14	16.8	20.1	30%
16	18.2	21.8	32%

Table 4. Time based production of cellulase in modified M9NG media using batch fermentation

Time (hrs.)	$O.D_{600nm}$	Wet cell weight g/L	Expression.
0	0		-
2	1.56	1.87	-
4	2.85	3.42	8%
6	5.8	6.96	10%
8	12.9	15.4	12.5%
10	23.4	28.0	14.5%
12	39.5	47.4	15.3%
14	46.5	55.8	20%
16	48.3	57.9	25%

Samples were collected after 4 hours of induction and protein expression was analyzed by using SDS-PAGE analysis.

Expression Analysis of the Recombinant Enzyme

Sample preparation for SDS-PAGE Analysis

The microfuge tubes were taken and 1ml of the respective induced samples were added in separate tubes. At 10,000rpm, centrifugation (Sigma, Germany, 1-14) was performed for 10 mint. The supernatant was wasted and 500µl of 20mM of Tris-base (pH 7) was added in a microfuge tube for pellet resuspension. Then SDS-PAGE analysis was performed and samples were prepared by taking 80µl of sample and 20µl of 5x sample loading dye. Both samples were syringed 20 times and then heated for five mints in a water bath at 1000C. A 12% SDS-PAGE gel was prepared by taking 3.3ml of distilled water, 4ml 30% acrylamide solution, 2.5 ml of 1.5M Tris-base pH 8.8, 100µl 10% SDS solution, 150µl 10%APS and 7µl TEMED. After that, resolving gel mixture was transferred into the gel castle, some portion of the gel castle was left for stacking gel and the water was overspread above the resolving gel in order to form a smooth surface. The gel was placed

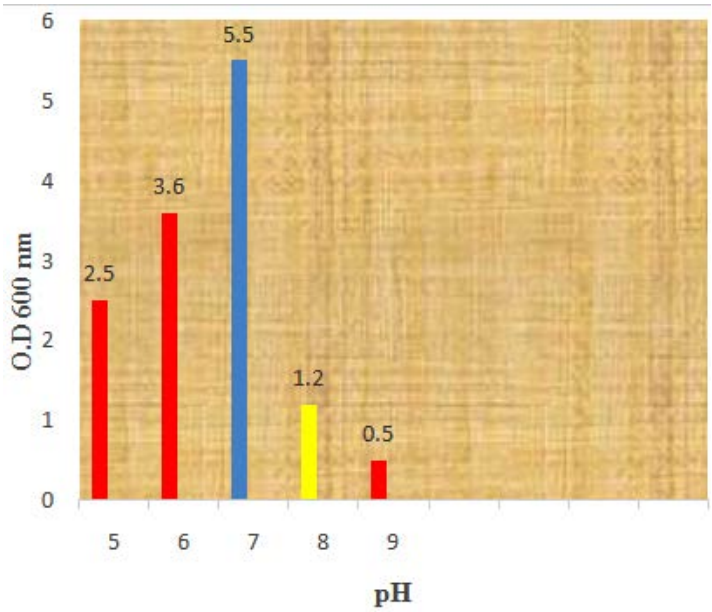

Fig. 5. Effect of pH on the cell growth

Table 5. Auto-induction based production of enzyme in LB media using batch fermentation

Time (hrs.)	$O.D_{600nm}$	Wet cell weight g/L	Expression.
0	0	0	-
2	0.25	0.3	-
4	0.65	0.78	7%
6	1.82	2.18	9.5%
8	3.9	4.68	12%
10	5.8	6.96	14%
12	9.5	11.4	15.5%
14	10.8	12.9	22.5%
16	11.2	13.4	30%

Table 6. Auto-induction based production of cellulase in modified M9NG media using batch fermentation

Time (hrs.)	$O.D_{600nm}$	Wet cell weight g/L	Expression.
0	0		-
2	1.3	1.56	-
4	2.4	2.88	2%
6	5.8	6.96	6%
8	11.5	13.8	9%
10	23.2	27.8	11.5%
12	36.8	44.1	16%
14	39.2	47.0	24%
16	41.3	49.5	30%

for some time and wait for polymerization. After the resolving gel polymerization, stacking gel was formed by taking 2.1ml of distilled water, 0.38ml of 0.5M Tris-base pH 6.8, 0.5ml of 30% acrylamide solution, 30µl 10% SDS, 30µl bromophenol blue, 30µl 10% APS and 4µl TEMED. The overlaid water was removed above the resolving gel and stacking gel was drained into the gel castle. The comb was put into the stacking gel and then left for polymerization. The comb was delicately extracted from the stacking gel without damaging the wells. The syringe was utilized for well washing and the gel castle was then placed in a gel tank. The electrophoresis tank and gel assembly castle was also filled with IX running buffer. The 15µl of each induced sample, 15µl of uninduced sample and 0.5µl ladder were loaded in the well. The gel was first electrophoresed at a voltage of 100V until the dye reached below the stacking gel and after that voltage was changed to 120V till the dye reached the bottom. In order to stain the gel, the coomassie brilliant blue R250 solution was used by placing the gel in it for 30 mints and then finally destained with a destaining solution.

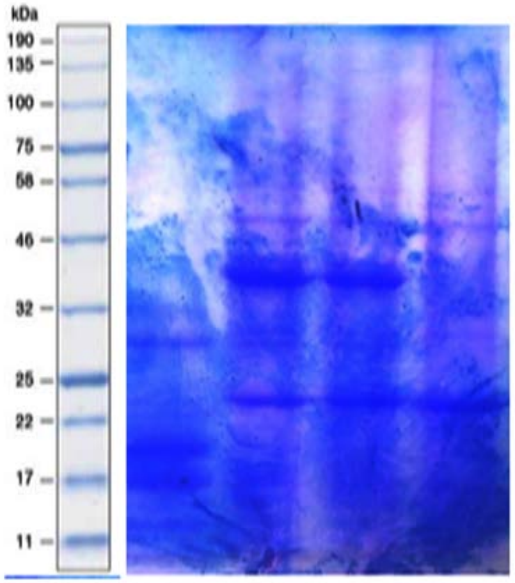

Fig. 6. 12% SDS-PAGE analysis of total cell proteins of *E.coli* BL21 after induction with lactose and IPTG. M: Marker, Row 1: lactose induction sample, Row 2: IPTG induction sample. Row 3: uninduced sample

Optimization of Conditions for Large Scale Production of Enzyme in Fermenter

Fermentation Conditions

Fermentation conditions were optimized for the maximum production of enzymes using batch culture. Agitation is one of the most important critical factors during fermentation. At the start of the batch, the stirring speed was adjusted at 400rpm and after the lag phase, the stirring speed was increased to 800rpm.The dissolved oxygen rate was optimized at 20%, 30%, and 40%. For maximum enzyme production, LB media and modified M9NG media were optimized. Inoculum of different strengths was used for the determination of maximum growth such as 1%, 5%, and 10%. Lactose was used as a source of cheapest inducer.

Batch Culture Fermentation

Time based induction

In the first experiment, 900ml of LB media was autoclaved.LB media containing 10% ampicillin was inoculated with 900ul of secondary inoculum and batch fermentation was performed. Fermentation conditions were adjusted according

Table 7. Auto-induction based production of cellulase in modified M9NG media using batch fermentation for 12 hours

Time (hrs.)	$O.D_{600nm}$	Wet cell weight g/L	Expression.
0	0		-
2	1.2	1.44	-
4	3.5	4.2	3%
6	6.8	8.16	8%
8	12.8	15.3	12%
10	24.7	29.6	20%
12	35.6	42.7	28%

Table 8. Auto-induction based production of cellulase in modified M9NG media. Using batch fermentation for 8 hours

Time (hrs.)	$O.D_{600nm}$	Wet cell weight g/L	Expression.
0	0	0	-
2	2.4	2.88	-
4	6.8	8.16	10%
6	14.5	17.4	16%
8	30.6	36.7	35%

to the optimized conditions as mentioned above. After 3 hours, the cells were induced with 10mM lactose and the total batch culture fermentation time was 16 hours. The samples were collected after 2 hours of interval.

In the second experiment, 900ml of modified M9NG media was autoclaved. M9NG media containing 100µg/ml ampicillin, 0.5%glycerol, 0.005% glucose, 0.025M K2HPO4, 0.025M NaH2PO4, 0.015M NH4Cl, 0.008M MgSO4.7H2O, 1mM trace metals was inoculated with 900ul of secondary inoculum and batch fermentation was performed. After 3 hours, the fermentation growth media was inoculated with 10mM lactose and the total batch culture fermentation time was 16 hours. Samples were collected after two hours of interval.

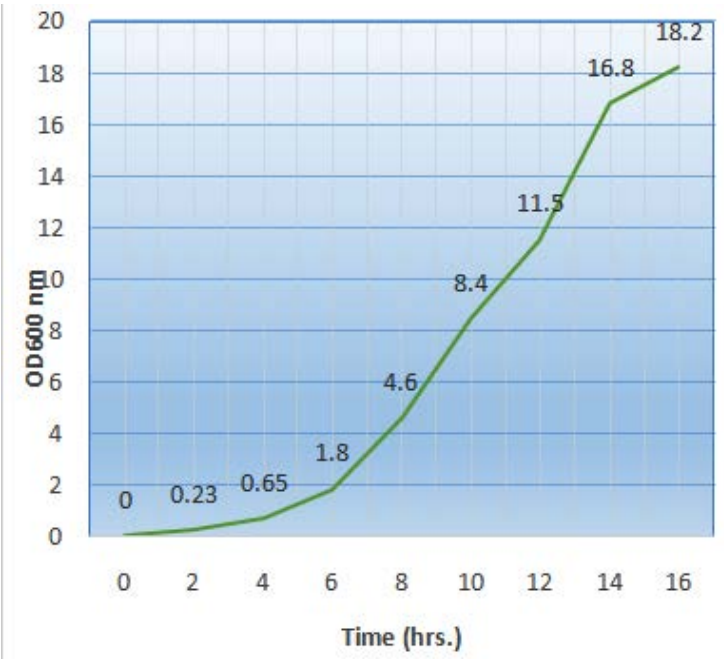

Fig. 7. Time based induction effect of time on the cellulase expression in LB media using batch fermentation

Table 9. Preparation Of dilutions For BSA Standard Curve

Sr No.	BSA (µg/ml)	Distilled water	Bradford Reagent
0	0	2.4ml	0.6ml
1	30µl	2.37ml	0.6ml
2	60µl	2.34ml	0.6ml
3	90µl	2.31ml	0.6ml
4	120µl	2.28ml	0.6ml
5	150µl	2.25ml	0.6ml
6	180µl	2.22ml	0.6ml
7	210µl	2.19ml	0.6ml
8	240µl	2.16	0.6ml
9	270µl	2.13ml	0.6ml
10	300µl	2.1ml	0.6ml
11	320µl	2.07ml	0.6ml
13	360µl	2.04ml	0.6ml

Table 10. BSA Standard Curve Absorbance Measurements

BSA (µg/ml)	A_{595nm}
0	0
30	0.115
60	0.197
90	0.286
120	0.392
150	0.468
180	0.582
210	0.675
240	0.778
270	0.886
300	0.972
330	1.14
360	1.25

Auto-Induction

In the first experiment of auto-induction, 900ml of LB media was autoclaved. LB media containing 10% ampicillin was inoculated with 900ul of secondary inoculum and auto-induced the cells with 10mM Lactose at the start of the fermentation and fermentation conditions were adjusted according to the optimized conditions as mentioned above. The total batch culture fermentation time was 16 hours. The samples were collected after 2 hours of interval. While in the second experiment of auto-induction, 900ml of modified M9NG media was autoclaved. Modified M9NG media containing 100µg/ml ampicillin, 0.5%glycerol, 0.05% glucose, 0.025M K2HPO4, 0.025M NaH2PO4, 0.015M

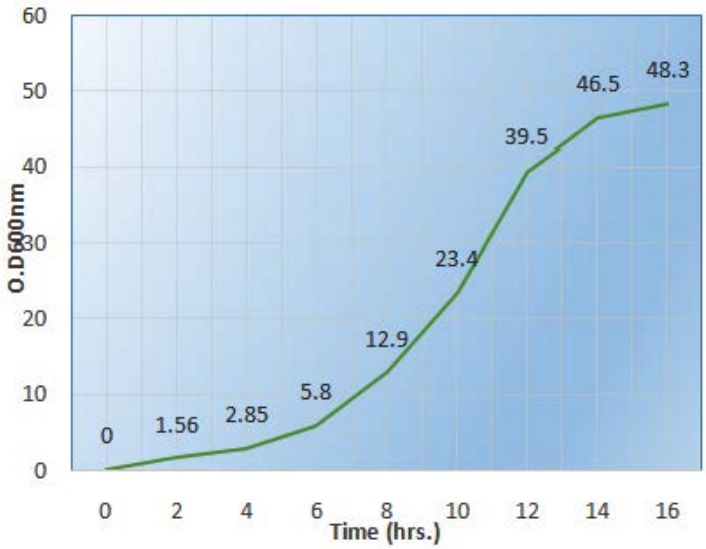

Fig. 8. Time based induction effect of time on the cellulase expression in modified M9NG media using batch fermentation

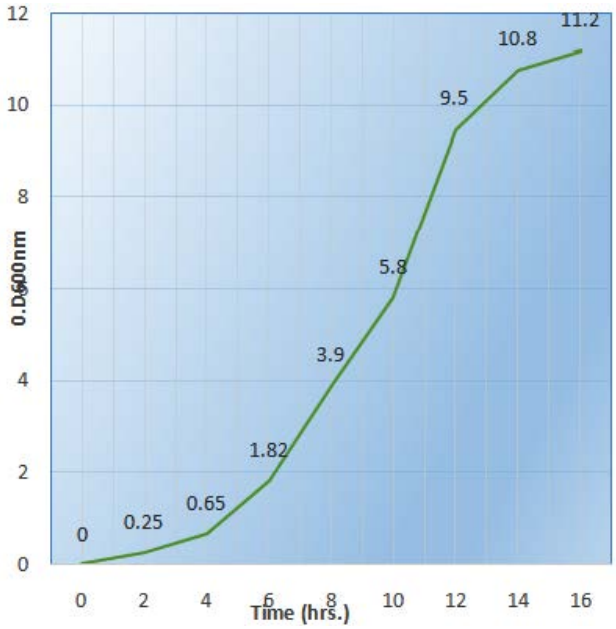

Fig. 9. Auto-induction based effect of time on cellulase expression in LB media using batch fermentation

NH4Cl, 0.008M MgSO4, 1mM trace metals were inoculated with 900ul of secondary inoculum and auto-induced the cells with 10mM Lactose and fermentation conditions were adjusted according to the optimized conditions as mentioned above. The total batch culture fermentation time was 16 hours. The samples were collected after 2 hours of interval.

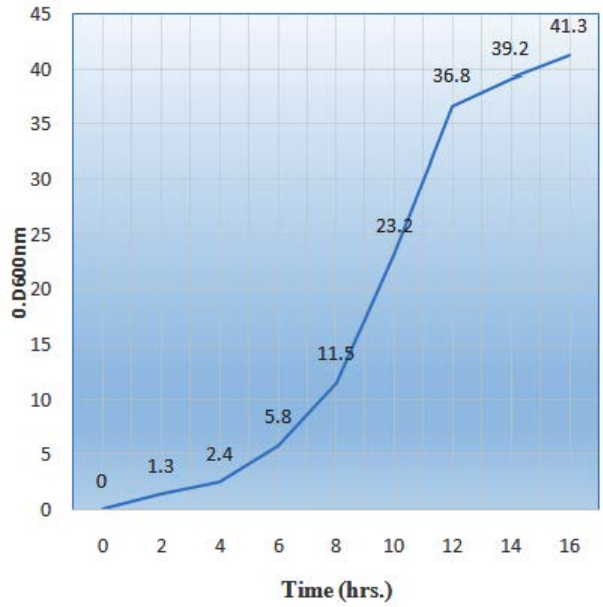

Fig. 10. Auto-induction based effect of time on cellulase expression in modified M9NG media using batch fermentation

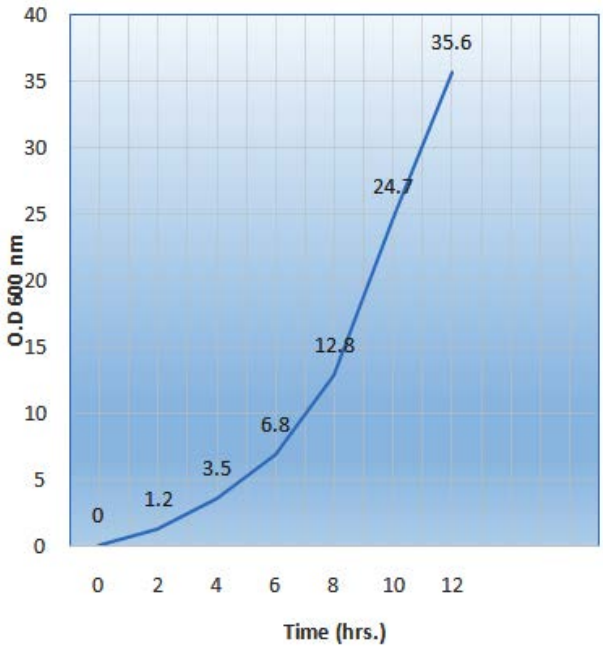

Fig. 11. Auto-induction based effect of time on cellulase expression in modified M9NG media using batch fermentation for 12 hours

In the third experiment of auto-induction, 900ml of modified M9NG media was autoclaved. Modified M9NG media containing 100µg/ml ampicillin, 0.5%glycerol, 0.005% glucose, 0.025M K2HPO4, 0.025M NaH2PO4, 0.015M NH4Cl, 0.008M MgSO4, 1mM trace metals were inoculated with 900ul of secondary inoculum and auto-induced the cells with 10mM Lactose and

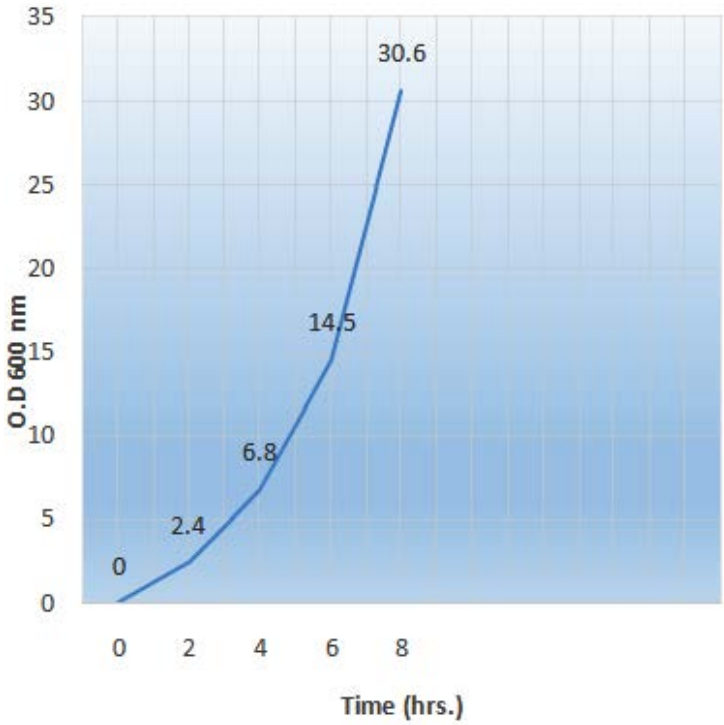

Fig. 12. Auto-induction Effect of time on the expression of cellulase in modified M9NG media using batch fermentation for 8 hours

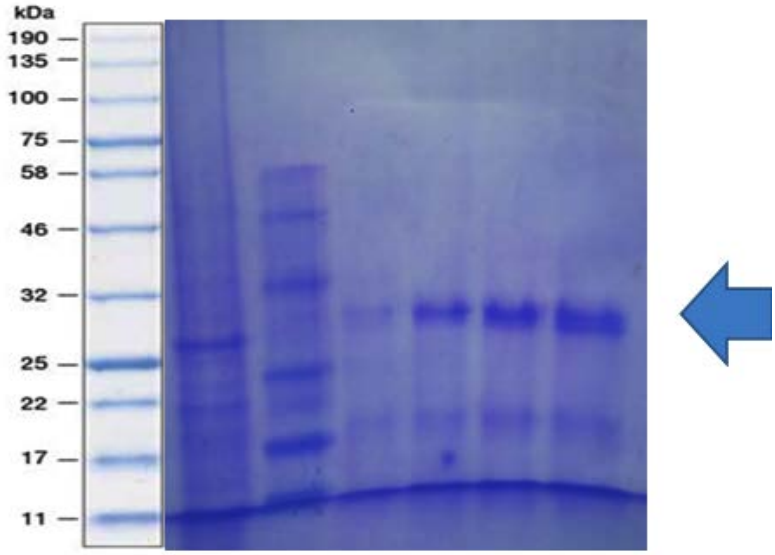

Fig. 13. 12% SDS-PAGE analysis of purified recombinant cellulase in modified M9NG media using batch fermentation for 8 hours. Row 1: uninduced sample. Row2: Marker. Row 3-6: induced samples after 2, 4, 6 and 8 hours of induction

fermentation conditions were adjusted according to the optimized conditions as mentioned above. The total batch culture fermentation time was 12 hours. The samples were collected after 2 hours of interval.

In the fourth experiment, 900ml of modified M9NG media was autoclaved. Modified M9NG media containing 100µg/ml ampicillin, 0.5%glycerol, 0.005% glucose, 0.025M K2HPO4, 0.025M NaH2PO4, 0.015M NH4Cl, 0.008M MgSO4, 1mM trace metals were inoculated with 900ul of secondary inoculum and auto-induced the cells with 1M Lactose and fermentation conditions were adjusted according to the optimized conditions as mentioned above. The total batch fermentation time was 8 hours. The samples were collected after 2 hours of interval.

Table 11.

Sample	Glucose Solution (1mM)	dH_2O	DNS ml	OD_{600nm}	µmole
1	20	980	3		20
2	40	960	3		40
3	60	940	3		60
4	80	920	3		80
5	100	900	3		100
6	120	880	3		120
7	140	860	3		140

BSA Standard Curve

BSA standard curve was determined by preparing a stock of 1µg/µl. 12 BSA dilutions were made having a total volume of 3ml. In each BSA dilution distilled water, BSA and Bradford reagent was added to make a total volume of 3ml. Each dilution was incubated at room temperature for 10 mints. Absorbance was taken at 595nm against blank consisted of distilled water and 0.6ml of Bradford reagent.

Glucose Standard Curve

For glucose standard curve, 10µl of 1oomM glucose was dissolved in 990µl of distilled water to make a stock of 1mM glucose. Seven glucose dilutions were made having a total volume of 1ml. After that, 3ml of DNS reagent was dissolved in each test tube and boiled at boiling

Table 12. Dilutions for enzyme activity

Sr.No	$O.D_{600nm}$	µmole/ml	DF* µmole/ml	U/ml	10µg/µl	U/µg
1	0.2335	1.3	10*1.3=13	1.3	1000	0.0013
2	0.211	1.1	50*1.1=55	5.5	200	0.02
3	0.149	0.7	100*0.7=70	7.0	100	0.07

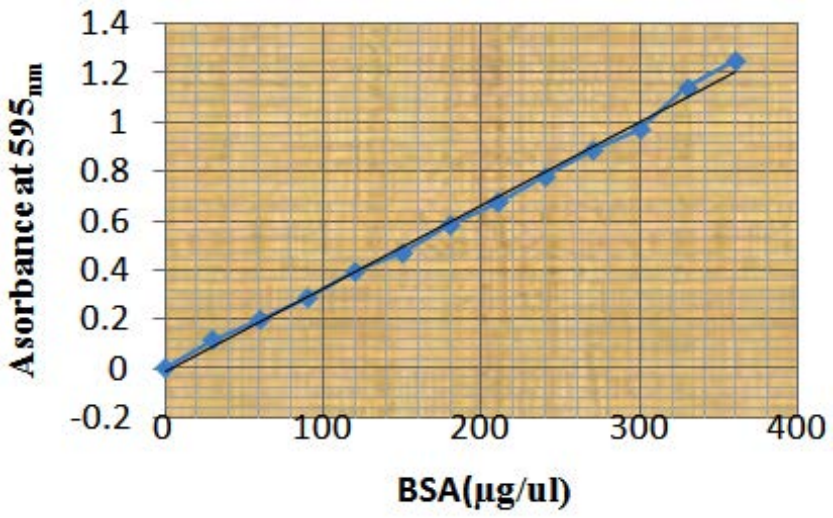

Fig. 14. Graphical representation of BSA standard curve

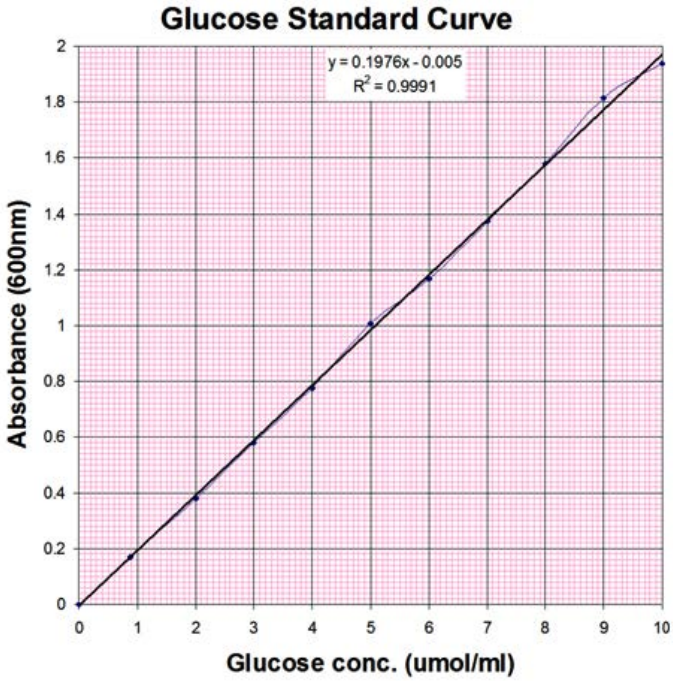

Fig. 15. Graphical representation of glucose standard curve

water for 15mints. Finally, absorbance was taken at 600 nm.

Enzyme Assay

Cmcase Assay

Cellulase activity was calculated by measuring reducing sugar that was discharged by a chemical reaction with dinitro-salicylic acid (DNS) reagent (Ghose, 1987). The original enzyme concentration was 10mg/ml. 500µl of 1%CMC was incubated with 10µl of diluted enzyme dissolved in 490µl of distilled water at 65oC for 10 mints. After, 3 ml of DNS reagent was added and heated the sample in boiling water for 15 mints. Absorbance was taken at 600 nm against the blank sample containing 500µl of CMC, 500µl distilled water and 3ml of DNS. The concentration of reducing sugar that was released by reaction with DNS was measured with a glucose standard curve.

RESULTS

Transformation of E.coli BL21 Codon Plus with Cloned pET22b (+)

After the transformation of E.coli BL21 Codon Plus with pET22b (+), the cells were spread on LB agar plates and placed in 37oC incubator for overnight. The result the of transformation shown was as below (Fig.2)

A single colony was transferred into the LB media from the transformed E.coli -BL21 Codon plus LB agar plate for screening purpose and the result was shown as below (Fig.3).

Optimum temperature was determined over a broad range of temperatures. At low temperature, the cellulase expression was low due to less microbial growth. The optimum temperature for the maximum cellulase expression and microbial growth was 37oC. At high temperature, the cells undergone the death phase due to which the expression was low (Fig.4)

Optimum pH was determined in the range of 5.0 to 9.0. The optimum pH for the cellulase was 7. The expression of cellulase was maximum at pH 7. The cellulase expression steadily declined with the rise or fall in pH beyond this range (Figure.5).

Expression analysis of cellulase enzyme

Transformed E.coli BL21 Codon plus cells were induced with lactose and IPTG in modified M9NG media for expression analysis of recombinant enzyme as mentioned in 2.4.1. When induction was done with lactose, sample was collected after 15-17 hours of induction. In

case of IPTG, the sample was collected after 4 hours of induction with IPTG . The recombinant enzyme was expressed according to the respective molecular mass of 62KDa (Figure.6).

Batch Fermentation

Time Based Induction

Time based induction was performed in LB and modified M9NG media. In this experiment, lactose was used as an inducer. After 4 hours, when the O.D reached 600nm the growth media was induced with lactose. Batch fermentation was performed for 16 hours and samples were collected after intervals At the end, the expression was checked. With the increase in time, the expression of cellulase was increased due to an increase in microbial growth. During this phase, expression was about 32%

During time based induction of batch fermentation in modified M9NG media, there was no expression due to less microbial growth. After the increase in wet cell weight, there was a notable increase in enzyme expression. Batch fermentation of 16 hours was performed. The expression was about 25%.

Auto-induction

In the first and second batch of auto-induction, LB and modified M9NG media were auto-induced with secondary inoculum. The lactose as an inducer was also added. Batch fermentation was performed for 16 hours. Samples were collected after intervals and expression was checked. (Table V, VI). During auto-induction based batch fermentation, the expression was about 30% using LB and modified M9NG media. The expression was almost the same in both media.

Auto-Induction Based Production of Cellulase in Modified M9NG Media using Batch Fermentation for 12 Hours

In the third batch of auto-induction, modified M9NG media was inoculated with secondary inoculum and auto- induced the media with lactose. Then batch fermentation was performed for 12 hours. When the batch fermentation was performed for 12 hours using modified, the expression was about 28% This expression was relatively low as compared to another batch of fermentation.

Auto-induction based production of cellulase in modified M9NG media using batch fermentation for 8 hours.

In the fourth experiment of batch culture fermentation, the cellulase expression was upto 35% in a minimum time period of 8 hours using modified M9NG media that was the optimized media for the production of cellulase in our research.

Expression Analysis of Purified Enzyme after Batch Fermentation

After batch fermentation, 12% SDS PAGE analysis was performed for investigating the recombinant enzyme expression. . The enzyme was expressed according to their molecular mass (62KDa).

BSA Standard Curve

In order to determine the enzyme concentration, the BSA standard curve was determined firstly.

For BSA standard curve, absorbance of different BSA (µg/ml) dilutions were taken for the construction of standard curve (Fig 14).

10 Glucose Standard Curves

In order to measure the enzyme activity, glucose standard curve was determined. By using 1mM glucose stock. In this experiment, 7 dilutions were made having a total volume of 1ml. Absorbance was taken at 600nm.

CMCase Assay

Cellulase activity was calculated by measuring reducing sugar that was released by reaction with dinitro-salicylic acid (DNS) reagent (Ghose, 1987). The concentration of reducing sugar that was released by reaction with DNS was measured with glucose standard curve. The enzyme activity was 7.0 U/ml

DISCUSSION

For numerous applications, industrial enzymes like cellulase, pectinases, etc are being utilized nowadays. One of the essential application is the creation of second-generation biofuels. The expanse of production must be small for these enzymes seeing the little value of the end product. Therefore, the use of expensive inducers and the inducible systems for producing these enzymes are undesirable (Munjal *et al*., 2012). Recombinant cellulase expression was analyzed in the E.coli BL21 codon plus using IPTG and lactose as an cheap source of inducer in LB or M9NG medium (Sadaf *et al*., 2007).In the present study,

recombinant cellulase was well expressed in E.coli BL21 according to their molecular weight (62KDa) using modified M9NG media. The expression level of the recombinant cellulase was round about 20% to 30%. The optimum temperature was 37oC for CMCase activity and cell growth (Kim *et al*., 2016). The Temperature of incubation shows a significant part in a microorganism's metabolic activities. In our conducted research, the observed optimum temperature for maximum cell growth was 37oC in the shake flask (Table 1). At high temperatures, cells can undergo the death phase. At 60°C and pH 7.0, the extracellular heat-stable cellulases showed the highest activity (Yang *et al*., 2010). The pH of the growth medium shows a significant part among physical parameters by prompting the morphological variations in microbes and secretion of enzymes. During microbial growth, the constancy of product in the medium is affected by the change in pH (Gupta *et al*., 2003). In our conducted study, the optimal pH of enzyme was measured at 5, 6, 7, 8, and 9. The optimum pH for the recombinant cellulase production was 7.0 when grown in batch fermentation (Table 2). At 60oC and pH 7, cellulases showed the maximum activity. Optimization of conditions is considered to be the most critical factor of fermentation at the commercial level production of enzymes. The expense of cellulases and their less productivity is a major limitation in the enzymatic saccharification of cellulosic constituents for fermentable sugars (Sukumaran *et al*., 2009). The aeration rate, agitation speed and inner pressure of bioreactor greatly affects the dissolved oxygen concentration in the medium (Seo *et al*., 2007). This change in the dissolved oxygen concentration results in the disturbance of microbial metabolites assembly and cell growth (Zhong, 2010).

In the present study, the optimal agitation speed and aeration rate for cell growth were 400rpm and 1.5 vvm. Batch fermentation was performed for different time periods using native LB as well as modified M9NG medium and lactose was used as a cheap source of inducer. Batch fermentation was performed for 16 hours in native LB and modified M9NG medium using lactose as a source of inducer as a result of which expression was minimum. The purpose of batch fermentation at different time periods using native LB and modified M9NG media was to optimized media and time of a batch under which maximum cellulase is produced in minimum time period. Production and expression of recombinant cellulase was maximum in auto-induced M9NG medium during the presence of specific salts and lactose was used as an inducer. Batch fermentation was performed for 8 hours. During lag phase in modified M9NG media, cell growth was negligible and steadily increased after entering the log pass till 8h. Expression level was upto 35% and cellulase production was enhanced six times more as compared to wild type strain.

CONCLUSION

Cellulases are being produced by utilizing several carbon sources, but these sources are highly costly. The use of cheap carbon sources can reduce the cost of cellulase. During this study, optimum conditions e.g. media, temperature, pH, inducer, time, carbon sources, nitrogen sources and agitation speed were optimized. The glucose, and glycerol were utilized as a cheap source of carbon. The lactose was used as the cheapest inducer. The modified M9NG media was optimized for batch fermentation and recombinant cellulase production was enhanced about six times during batch fermentation in a minimum time period of 8 hours. The optimal pH and temperature for the cellulase was pH 7 and 37oC. The maximum enzyme activity was 7.0U/ml. Under optimized conditions, the cellulase production was enhanced six times more in comparison to remote type strain. The expression level of the cellulase was approximately 30% to 40%.

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