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Original Research Article

Production of thermoalkalistable microbial cellulases from some agricultural wastes under solid state fermentation conditions

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A B S T R A C T

Grinded palm leaf is an agricultural residue which has a high cellulosic content. This cheap biomass can be used for production of thermoalkalistable microbial cellulase for application in biodetergent formulations. Screening studies were carried out for one hundred fifty three bacterial isolates with respect to their ability to produce extracellular cellulase grown on grinded palm leaf as agro-industrial wastes at 55°C and pH9.0. The potent cellulase thermophilic produced bacterial isolate was identified as *B. stearothermophilus* KGKSA40. Effect of different physico-chemical parameters to achieve maximum cellulolytic enzyme production was systematically investigated. The overall steps protocol resulted in raising the purification fold to 20.48 times. Aspartic and glutamic acid gave the highest concentrations among the amino acid detected in the purified cellulase. Optimum incubation temperature was 50–55. The purified cellulase was thermostable with maximum activities at 50–55ºC. The optimum pH for maximal cellulase activity was 10 and pH stable at 10.5. The activities of the purified enzyme increased gradually with the increase of time up to 36 h incubation of the reaction mixture. The purified cellulase exhibited good stability towards chlorine. The purified cellulase showed that cellulase not only stable but enhanced maximal activity by certain agents. Compatibility of the purified enzyme with various commercial detergents reported that cellulase stable in presence of Ariel and Omo. The wash performing analysis of the present enzyme revealed that, it could effectively remove a variety of stains. The crude enzymes of this bacterial strain with a potential to be a candidate for the application in the detergent industry.

K e y w o r d s

Microbial enzymes, Thermoalkalistable enzymes, Biodetergent, Solid state fermentation, *Bacillus stearothermophilus*

Introduction

Plant cell walls containing cellulose, hemicellulose, pectin and lignin are the most abundant organic resource on the planet (Uffen, 1997). Enzymes are biological

catalysts which are the most remarkable, highly specialized and energized protein molecules found in every cell and are necessary for life. There are over 2000 known enzymes, each of which is involved with one specific chemical reaction. These proteins and their functions are determined by their shape. They have extraordinary catalytic power, often for greater than that of synthetic or inorganic catalysts (Sharada *et al.,* 2014; Mohanta, 2014).

Agro industrial waste like fruits and vegetables skins are thrown in the garbage or utilized as feed or fertilizer (Singh *et al.,* 2014). Cellulase is a class of enzyme that catalyzes the cellulolysis i.e., hydrolysis of cellulose. Cellulose is the major polymeric component of plant matter and is the most abundant polysaccharide on Earth. It is representing about 1.5×10^{12} tons of the total annual biomass production through photosynthesis especially in the tropics and is considered to be an almost inexhaustible source of raw material for different products (Sukumaran *et al.,* 2014). Successful utilization of cellulosic materials as renewable carbon sources is dependent on the development of economically feasible process technologies for cellulase production. It has been calculated that cellulase production accounts for ~27–20% of the cost of ethanol production from lignocellulosic materials. So, to keep the costs down, it is therefore very important to use a substrate that is inexpensive, such as wheat straw, rice bran, green gram husk, etc. Large numbers of microorganisms are capable of degrading cellulose, fungi and bacteria are the main cellulase producing microorganisms (Falkowski *et al.,* 2000; Lotfi, 2014).

Cellulase are important enzymes not only for their potential applications in different industries like industries of animal feed, agricultural, laundry and detergent, textile, paper and pulp, wine and brewery, food processing and olive oil extraction. Carotenoid extraction, pharmaceutical and medical sciences, analytical applications, protoplast production, genetic engineering and pollution treatment, but also for the significant role in bio conversion of agriculture wastes into sugar and bio ethanol (Han and Chen, 2010; Sharada *et al.,* 2014). The most abundant lignocellulosic agricultural wastes produced every year are corncobs, corn stover, wheat, rice, barley straw, sorghum husks, coconut husks, sugarcane bagasse, switchgrass, pineapple and banana leaves (Demain, 2005; Bisaria and Kondo, 2014).

In the present study, *B. stearothermophilus*-KGKSA40 was isolated from soil samples which have the ability to hydrolyse different cellulosic substrates by producing of multiple cellulolytic enzymes under solid state fermentation condition. Various physico-chemical parameters were optimized to achieve maximum enzymes production. Purification of the produced cellulase under all optimal conditions was also investigated, characteristics of the purified cellulase and its application in bio-detergent formulations.

Materials and Methods

Construction of cellulase standard curve

A stock solution (10.000 µg/ml) of the purified cellulase (Sigma) was prepared in a phosphate buffer at pH 9.0. After preparing the required dilutions for cellulase, only 0.1 ml of each dilution was transferred to each well in the assay plate. Incubation was performed at 55ºC for 20h. Then mean diameters of clearing zones (mm) were determined. One unit is defined as the amount of enzyme protein (mg) required to exert one unit of clearing zone (mm) in one

unit of time under all the specified conditions of enzyme assay (clearing zone technique).

Standard curve for protein determination: Protein of enzymatic preparation of cellulase was determined by the method of Lowry *et al.,* (1951).

Nutrient agar (NA) medium: This medium contained (g/l): Peptone, 5; sodium chloride, 5; beef extract, 3; agar-agar, 15 and distilled water up to 1000 ml. The ingredients were dissolved by heating; pH was adjusted at 7 and sterilized at 121ºC for 15 min (Shiriling and Gottlieb, 1966).

Wastes: The grinded palm leaf wastes were collected from different restaurants in El-Khormah, Taif, KSA. These wastes were washed, dried in open air and then grounded for application in the production media. Bagasse, rice straw and water hyacinth were collected, dried and prepared in the form of a ground preparation.

Production media: The basal medium (BM) was prepared according to Vincent (1970). It contained the following (g/l): Sucrose, 10; KNO_3, 0.6; KH_2PO_4, 1; $MgSO_4$, 0.25 and $CaCl_2$, 0.1 was found most convenient for the production of different enzymes. It was modified to include the following constituents: (g/l) NaCl, 6; $(NH_4)_2SO_4$, 1; yeast extract, 1; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.1; $CaCl_2$, 0.1 and distilled water up to one liter.

Grinded palm leaf basal medium (GPLBM): It contained the same constituents of BM supplemented with grinded palm leaf (5%, w/v). After incubation period 10 ml of distilled water was added for enzyme extraction.

Isolation of thermophilic bacteria: Thermophilic bacterial isolates were isolated from different localities representing desert soil samples collected from different localities of Khormah governorate, Taif, KSA. Soil samples were applied using the soil dilution plate technique.

IX-Qualitative screening test media, methods and conditions: The basal medium used in this assay was supplemented with 1% cellulose and 1.5% agar.

Media used in screening test: For detecting the cellulase activities of all best enzyme producers on different agro-industrial wastes. The previously mentioned BM was used, pH was adjusted at 9.0. Only 1 g of used waste was wetted by 10 ml of broth BM in the baxter bottle of 100 ml capacity. The baxter bottles were autoclaved at 121ºC for 15 min. All thermophilic bacterial isolates were subcultured onto nutrient agar slants. After incubation at 55ºC for 24 h the growth was scratched with 5 ml of sterile saline solution (0.9 % NaCl). Each flask was inoculated with 1 ml of bacterial suspension, and then incubated at 55ºC for 48 h. The cell-free filtrate (CFF) of each baxter bottle was prepared, then cellulytic activities were detected by cellulose clearing zone (CCZ) technique.

Assay medium: The cellulose activity medium contained 1% (w/v) crystalline cellulose powder with 1.5% agar-agar for solidification to be dissolved up to 100 ml of Tris-buffer 0.2M at pH 9. After flooding the plates by Lugol's iodine solution, the mean diameters of clearing zones were calculated to express the amount of cellulase activity.

Identification of the potent thermophilic bacterial isolates: The potent bacterial isolate was identified by examination of their morphological physiological and biochemical characteristics according to

Schallmey *et al.*, (2004); Collee *et al.*, (1996); Hensyl (1994); Barrow and Feltham (1993) and Parker (1984).

Preparation of cell-free-filtrate (CFF): This was performed by preparing the previously mentioned production media. At the end of incubation period, 10 ml of distilled water were added in the case of detection of cellulase were used as a substrate, then, the bacterial growth was harvested by filtration for separate large particles of two wastes by using gauze piece. The filtrates were centrifuged at 5,000 rpm for 20 min. The supernatant was obtained and preserved into the refrigerator as a crude enzyme, and/or assayed at the same times.

Parameters controlling the thermoalkalistable cellulase productivity

(1) Temperatures: The potent isolate was allowed to grow on GPTBM at different temperatures for 48 h. (2) pH values: The pH was adjusted at different pH values using 6N NaOH and 6N HCl. (3) Substrate concentrations: Different concentrations of substrate [g/bottle (20 ml), w/v] were applied. (4) Carbon sources: Different carbon sources were introduced into the production medium at an equimolecular level located at 1% (w/v) glucose. The organic carbon sources were introduced at the level of 1% (w/v). (5) Nitrogen sources: Production medium was supplemented with different nitrogen sources with an equivalent amount of nitrogen that present in ammonium sulphate (0.1%; w/v) in basal media. Peptone was introduced as organic nitrogen source at the level of 0.1%. (6) Inocula sizes: Different inocula sizes of heavy spore suspension of the bacterial isolate (prepared by washing each slant by 5 ml of sterile saline solution under aseptic conditions) were used. (7) Incubation periods: The potent bacterial isolate was incubated for different incubation periods. (8) Metallic ions: The supplemented metal ions were in the form of EDTA, $ZnCl_2$, $CdCl_2$, $CoCl_2$, $FeCl_3$, and sodium selenite. (9) Vitamin requirements: Different vitamins were added separately to the production media specialized at 100 ppm. (10) Amino acids: This experiment was controlled by performing of parallel one containing the original nitrogen source i.e. ammonium sulphate. (11) Incubation conditions: This was carried out by incubation the flasks containing the production media on the shaker 50 rpm/min, while another flasks were incubated under static conditions at 55ºC. (12) Bottle capacities: It was performed by using five different capacities for agricultural wastes as substrates.

Purification of thermoalkalistable cellulase: Strain B-78 was allowed to grow under the optimal static natural substrate under solid state fermentation conditions. The bacterial growth was harvested by centrifugation at 5,000 rpm for 15 min. The supernatant was filtrated, and the obtained cell-free filtrate was preserved in the refrigerator as a crude enzyme according to Bayoumi and Bahobil (2011). The chart of Gomori (1955) as mentioned by Dixon and Webb (1964) was applied to calculate the solid ammonium sulphate. The procedure was performed by introducing the obvious precipitate (Selected ammonium sulphate ppt. in solution after dissolving it in the Tris-buffer at pH 9.0) into a special dialysis plastic bag for dialysis against distilled water for 12 hours. The obtained enzyme preparation was concentrated against sucrose to be available for the application in column chromatographic technique. The dialyzed-partially purified-enzyme preparation was applied onto a column packed with Sephadex G 200. From the relationship plotted previously after

applying Sephadex G-200 column chromatographic technique, the fractions which gave the highest specific activity among all other fractions (i.e. the highest peak of each enzyme) were collected and concentrated to the least volume (1ml) and then applied into a second column (Pharmacia 2.5 x 50 cm), packed with Sephadex G-100.

Amino acid analytical data of the purified enzyme: The hydrolyzed protein amino acids have been determined in the central Lab. for Food, Agricultural Research Center according to the methods described by Pellet and Young (1980).

Factors affecting the purified thermostable cellulase activity

(1) Incubation temperatures: This was carried out by incubation of enzyme after pouring in assay media at different incubation temperatures. (2) Heat stability: This was carried out by incubating the purified enzyme for 2 h at different temperatures. (3) pH values: The purified enzyme was incubated at different pH values by using phosphate, Tris- or Glycine buffers. (4) pH stability: This experiment was carried out by mixing equal amounts of buffer at different pH values with purified enzymes, followed by incubating the mixture for 3h at 55ºC. The different values of pH used before assay. (5) Incubation periods: It was performed as follows: after pouring the purified enzyme in assay medium, plates were incubated at 55ºC and enzyme activity was assayed at different incubation periods. (6) Enzyme concentrations: The purified enzyme was applied at the following concentrations 25, 50, 75, 100, 125, 150, 175, and 200 µl into the assay media plates. All concentrations were incubated at 55 ºC for 18 h. (7) Substrate concentrations: This was carried out by applying cellulose as substrates at concentrations of: 0.1, 0.2, 0.5, 1.0, 2.5 and 5.0 (% w/v). (8) Stability with chlorine: This was carried out according to Singh *et al.* (1999). The purified enzyme was incubated with different concentrations of chlorine viz. 5, 7, 10, and 15, µl/ml, then the enzyme was inoculated into assay media plates at different time intervals viz. 15, 30, 45 and 60 min. (9) Oxidizing agents and surfactants: This was carried out according to Moreira *et al.,* (2002). The cellulase was incubated with different concentrations of some oxidizing agents and surfactants viz., sodium dodecyl sulphate (SDS), sodium perborate, hydrogen peroxide, tween 20, tween 40, tween 80, triton X100 and sodium cholate at 1, 2, 3, 4, 5, 7.5 and 10 (% v/v or w/v) for 2 hours. (10) Compatibility with various commercial detergents: Rabso, Ariel, Omo, Lang, Persil, Xtra, Ariel automatic, and Xtra automatic were supplemented separately to cellulase in a concentration of 7 mg/ml, while the liquid detergents viz., General and Pril were added in concentration of 7 µl/ml.

Results and Discussion

Qualitative screening test for selection of the potent thermostable enzymes producers: One hundred fifty three theromophilic bacterial isolates were isolated from different localities of different soil and water samples collected from Khormah governorate, Taif, KSA. These isolates were purified, and subjected to a screening program in order to evaluate their cellulytic activities by measuring (observing) the hydrolysis of cellulose around the bacterial colonies. Out of 153 isolates there was found that out of 20 isolates gave a cellulytic activities, only 5 strains are considered a best cellulase producers compared with other isolates.

Quantitative screening for selection of the potent enzymes producer: This experiment was carried out to determine the potent

enzyme producer bacterial isolates on the basis of mean diameters of clearing zones (mm) by testing the potency of the best bacterial isolates producers selected from the previous test to attack some agriculture and industrial wastes. Bacterial isolate number KGKSA 40 gave higher cellulase productivity by attacking grinded Palm leaf, compared to other wastes and other isolates, where it reached up to 23.5 and 27.8 (mm).

Identification of the potent bacterial isolates: The potent bacterial isolate KGKSA40 was subjected to an identification program to the species level. All morphological characteristics and stain reaction led to the fact that the potent bacterial isolate under identification was suggestive of being belonging to the genus *Bacillus*. Since this bacterium has the ability to grow up to 65 ºC, it is suggestive of belonging to the species *stearothermophilus* according to Bergey's Manual of Systematic Bacteriology (Sneath, 1986). It could be given the tentative name *Bacillus stearothermophilus* KGKSA40.

Parameters controlling the four thermostable enzymes production

Different incubation temperatures: Maximum cellulase productivities was at 55˚C where it reached up to 0.18 (U/ ml) (Table 1).

Effect of different pH values: Maximum cellulase productivity reached up to 0.21 U/ml by *B. stearothermophilus*-KGKSA40 incubated at 55ºC was pH 9.0 (Table 1).

Different substrate concentrations: Maximum cellulase productivity reached up to 0.1850 U/ml at grinded Palm leaf concentration of 2 g/baxter bottle (Table 2).

Application of different carbon sources: Starch and cellulose was the best carbon source for cellulase production since the maximum yield of cellulase reached up to 0.2960 and 0.281 U/ml, respectively (Table 3) and ammonium sulphate increased the cellulase productivity (Table 3).

Different inocula sizes and incubation periods: Maximum cellulase activity reached up to 2.160 U/ml by *B. stearothermophilus*-KGKSA40 at an inoculum size 1.5 ml /baxter bottle at 55 ºC (Table 4). *B. stearothermophilus*-KGKSA40 were able to grow on grinded Palm leaf and produces the highest yield of cellulase productivity reached its maximal value 1.45 U/ml at the end of 48 h incubation period (Table 4).

Different metallic ions concentrations: None of the tested metal ions exerted any inductive effect on cellulase produced by *B. stearothermophilus*-KGKSA40 (Table 5).

Effect of different vitamin requirements application: Maximum cellulase productivity was in the absence of any of the tested vitamins (Table 6).

Application of amino acids: Various amino acids succeeded to promote the biosynthesis of cellulase by *B. stearothermophilus*-KGKSA40 where out of 18 amino acids used, the four amino acids exhibited high stimulatory effects on cellulase productivity were DL-aspartic acid, L-proline, glycine, and DL-tyrosine. Aspartic acid was considered the best inducer (Table 7).

Effect of shaking and static conditions: Static conditions gave the highest yield of cellulase which reached its maximal value 2.22 U/ml for *B. stearothermophilus*-KGKSA40 (Table 8).

Effect of different bottle capacities: The optimum bottle capacity for cellulase production by *B. stearothermophilus*-KGKSA40 was found to exhibited its highest cellulase yield with 500 ml bottle capacity where its productivity reached up to 3.9U/ml

(Table 9). A summary of the optimal nutritional and environmental conditions for thermostable hydrolytic enzymes production by *B. stearothermophilus*-KGKSA40 grown on grinded palm leaf wastes as preferable fermentable substrates was given in Table 10.

Purification of cellulase produced by *B. stearothermophilus*-KGKSA40 allowed to grow on grinded palm leaf under solid state fermentation: Cellulase produced by *B. stearothermophilus*-KGKSA40 previously grown on grinded Palm leaf as a preferable substrate supplemented with mineral salts under the optimum nutritional and environmental conditions recorded in Table 10 were purified to homogeneity as previously mentioned, by performing ammonium sulphate fractionation, dialysis, and applying column chromatography on Sephadex G200 and G100.

Enzyme production and preparation of CFF: At the end of incubation period, 500 ml of cellulase enzymes media were extracted and collected separately. Centrifugation of the obtained extracts was done at 5000 rpm for 15 min at 10ºC. The precipitate was collected and tested for determination of enzyme activities and protein content and corresponding specific activity was calculated.

Fractional precipitation by ammonium sulphate: The most active enzyme protein preparation was obtained at an ammonium sulphate level at 80 % for cellulase where the activity was reached up to 2.66 U/ml and protein content 1.35 mg /ml. Only 50 ml were obtained at the end of the process of dialysation against tap water in case of cellulase.

Concentration by dialysation against sucrose: The most active ammonium sulphate fractions previously obtained at the best saturation, (50 ml) in case of cellulase, were dialyzed against distilled water followed by dialysis against sucrose crystals until a volume of 4 ml was obtained and specific activity was determined as 5.45 U/mg^{-1} protein.

Purification of cellulase by Sephadex G-200 column chromatographic technique: Fifty fractions of cellulase were collected and the highest fractions activities appeared from fraction (15–25) where many peaks occur and the fraction number (20) reached the highest specific activity up to 31.125 U/mg^{-1} protein.

Purification of cellulase by Sephadex G-100 column chromatographic technique: A sharp one peak was obtained in all cases of the four enzymes purifications and specific activities reached its maximal value up to 36.88 U/mg^{-1} protein for cellulase. A summary of the purification steps of cellulase produced by *B. stearothermophilus*-KGKSA40 (Table 11).

Amino acids analysis of the purified enzymes: Fourteen amino acids were detected in addition to ammonia in the purified cellulase produced by *B. stearothermophilus*-KGKSA40 and the acidic amino acids (glutamic, and aspartic acids) gave the highest concentrations among all the amino acids detected. Interestingly, it could be noticed that the glutamic acid and aspartic acid were occurred in all enzymes in high concentrations (Table 12).

Characterization of the purified enzymes produced by *B. stearothermophilus*-KGKSA40 at 55ºC: The aim of the present series of experiments was to investigate some properties of the partially purified enzyme produced by *B. stearothermophilus*-KGKSA40 allowed to grow on grinded Palm leaf as best substrates and incubated under all optimal nutritional and

environmental solid state fermentation conditions..

Incubation temperature: Maximum cellulase activity produced by *B. stearothermophilus*-KGKSA40 was at 50–55ºC, where it reached up to 0.77 U/ml (Table 13).

Table 13 shows different incubation temperatures in relation to the activity of purified cellulase.

Thermostability: Purified cellulase exhibited its maximum activity at 50–55ºC, where it reached up to 0.777 U/ml (Table 14).

pH values: Purified cellulase exhibited the maximum activity at pH 9 of Tris-HCl buffer and gave 0.75 U/ml (Table 15).

pH stability: The purified cellulase activity reached its maximal value up to 0.775 U/ml at pH 10.5 (Table 16).

Incubation periods: Highest cellulase activity was obtained after 36h incubation time and reached up to 1.91 U/ml. The catalytic action of the enzymes was increased by increasing time intervals applied in this study Table 17.

Purified enzyme concentrations: Results recorded that continuous increasing of enzyme activity due to the increase of enzyme concentration units, where it reached up to 1.51 units/ml for the purified cellulase as shown in Table 18.

Substrate concentration: Maximum cellulase activity was attained at 0.2% substrate (cellulose) concentration where it reached up to 0.75 U/ml, and the concentration from (0.5–2.5 %) exhibited an equal activities i.e., 0.61 U/ml (Table 19).

Stability with chlorine (Na-hypochlorite): Chlorine concentration up to 7µl/ml of purified cellulase was stable up to 10µl chlorine/ml of enzyme, and even increasing this concentration up to 15µl/ml the remaining activity was 56–2 % (Table 20).

Oxidizing agents and surfactants: Purified cellulase was stable towards SDS, H_2O_2, Tween 20,Tween 80 and Triton X100 and exerted a high stability at all tested concentrations (i.e., up to 0.5 % w/v or v/v). However, maximum stimulation was in the presence of H_2O_2 at 1–2, 5–7.5% v/v and Tween 20 at 3–4 % v/v. On the other hand, higher concentrations (7.5–10 % v/v or w/v) of Tween 40 and sodium cholate exerted decreasing activity.

Compatibility of the purified enzymes with various commercial detergents: Data recorded in Table 21 indicated that the cellulase enzyme reached its maximum activity in the presence of Ariel detergent where, the enzyme activity increased up to 130 %. It was found that the enzyme exhibited its minimum activity in the presence of Persil and Lang, while, in presence of the other applied detergents, there was an acceptable activity retained.

One of the main goals of enzymes research is industrial applications. Nowadays, we are surrounded by enzymes as well as chemicals produced by enzymes in our daily life. The majority of the industrial enzymes are of microbial origin**.** The major goals for future cellulase research would be: (1) reduction in the cost of cellulase production and (2) improving the power of cellulase to make them more effective, so that less enzyme is needed. Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Habitats where these substrates are present are the best sources for isolation of cellulolytic microorganisms.

One hundred fifty three bacterial isolates were isolated from different soil and water samples collected from different localities in Khormah governorate, Taif, KSA. These bacterial isolates were grown at 55°C and at pH 9.0 to be able to produce a thermostable and alkalophilic cellulase which favorable to be used as additive to bio-detergent formulations. A screening test of cellulytic productivities of all bacterial isolates resulted in the fact that, five bacterial isolates gave highest cellulase productivity.

From industrial point of view, in order to production of low cost of enzymes, this bacterial isolate KGKSA40 under study were allowed to grow on natural substances such as grinded palm leaf under solid state fermentation (SSF). However, the selection of the previously mentioned substrate for the process of enzymes biosynthesis was based on the following factors viz (i) they represent the most cheapest agro-industrial wastes in Khormah governorate, Taif, KSA; (ii) they are available at any time of the year; (iii) Their storage represents no problem in comparison with other substrates and (iv) they resist any drastic effect due to the exposure to other environmental conditions e.g. temperature, variation in the weather from season to season and or from day to night.

The enzymatic hydrolysis of cellulosic feedstock has several advantages, namely mild experimental conditions, less energy consumption and avoidance of pollution over the chemical processes (Saratale *et al.,* 2008, 2010). Optimal parameters of the alkaline-thermostable enzymes biosynthesis from microbial origin varied greatly, with the variation of the producing strain, environmental, and nutritional conditions**.** The production of microbial cellulase has long been known to be influence by different factors like the strain type, reaction conditions and substrate types, since the relationship of these factors might affect the production of the cellulase enzymes (Zhang *et al.,* 2006; Ram *et al.*, 2014; Yassien *et al.,* 2014; Jabasingh *et al.,* 2014; Dabhi *et al.,* 2014). Also, the optimization of the production of enzymes is important for increasing productivity and reducing costs strategies.

Incubation temperature plays an important role in the metabolic activities of a microorganism. Even slight changes in temperature can affect enzymes production. Temperature control is very important for fermentation processes, since growth and production of the microbial enzymes are usually sensitive to high temperature. It must be noted that, the optimum temperature for the production of an enzyme does not always concide with that for growth (Dabhi *et al.,* 2014).

In the present study the optimum incubation temperature by *B. stearothermophilus*-KGKSA40 was 55°C. The thermo stable nature of cellulolytic enzymes added advantages to the lignocelluloses bioconversion processes so that it remains viable and active at higher temperature. At higher operation temperature of stability of the enzymes significantly influence the bioavailability and solubility of organic compounds. Because of these enzymes can efficiently degrade of cellulosic biomass and increased flexibility with respect to process configuration, and overall improves the economy of the process (Viikari *et al.,* 2007). The thermo stability of this enzyme indicates industrial applicability in the food, sugar, fuel ethanol and agricultural industries where process operation applied with higher temperature (Jang and Chen, 2003; Ram *et al.,* 2014; Yassien *et al.,* 2014; Jabasingh *et al.,*2014; Dabhi *et al.,* 2014).

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium (Gupta *et al.*, 2003). The optimal pH varies with different microorganisms and enzymes. The initial pH of fermentation medium has a significant effect on bacterial growth and enzyme production. The pH of fermentation medium modulates microbial growth and enzyme production (Odeniyi *et al.,* 2009). The cellulase activity was affected by the pH and temperature of the reaction mixture (Yin *et al.,* 2010).

One of the most fascinating data, the optimum pH value for the growth of thermoalkalophilic bacterial strain viz. *B. stearothermophilus*-KGKSA40 was at pH 9 for all the produced thermostable cellulase. Interestingly, the maximum cellulase production was attained at pH 9 for both thermoalkalophilic bacterial strains viz. *B. stearothermophilus*-KGKSA40 with grinded Palm leaf by using HCl and NaOH in the production media.

The optimum substrate concentration was 2 g/baxter bottle (7.5%) from cheapest natural solid substrate grinded Palm leaf stimulated the highest yield of thermoalkalophilic cellulase production. This means simply that, this solid natural substrate fulfilled both the energy and nutritional requirements for *B. stearothermophilus*-KGKSA40 to be able to biosynthesize the highest yield of thermoalkalophilic cellulase under solid state fermentation conditions. The highest enzymes productivity was followed by a decline at higher concentrations. It may be due to the substrate thickening of the production fermentation medium that result in bad mixing of air which is essential for the growth of organism, and subsequently the production of enzyme and interfere with O_2 leading to limitation of dissolving oxygen for the growth of bacteria.

The best substrate (Grinded Palm leaf) concentration that fulfilled the highest yield of cellulase it was found that, 2 g/baxter bottle (7.5%) of *B. stearothermophilus*-KGKSA40. Zhang *et al.* (2006) showed a positive correlation between the production of cellulase enzyme from cellulolytic microorganisms and different composition of cellulose, hemicelluloses and lignin in different cellulosic materials.

Different organic and inorganic nitrogen sources were tried to improve cellulase production. Organic nitrogen showed superiority over inorganic nitrogen sources for the production of enzymes (Fawzi *et al.*, 2011). In a trial to study the effect of introducing some carbon sources on the alkaline thermostable enzyme(s) production, it was found that, all the tested carbon sources failed to induce alkaline-thermostable amylase production by growing *B. stearothermophilus*-KGKSA40 on grinded Palm leaf under SSF conditions.

Alkaline-thermostable cellulase productivity, it was found that, cellulose and starch and cellulose was the best carbon source for *B. stearothermophilus*-KGKSA40 allowed to grow on grinded Palm leaf. Ammonium sulphate induces the alkaline-thermostable cellulase production by *B. stearothermophilus*-KGKSA40.

The best incubation period for alkaline-thermostable cellulase production on grinded palm leaf by *B. stearothermophilus*-KGKSA40 was found to be 48 and 72h respectively. Influential studies of metal ions on enzyme are very important because it increases enzymes industrial applications. It has been previously reported that some metals acts as a cofactor of cellulase, inducing or inhibiting the amino acids of the

active site of the enzymes (Ram *et al.,* 2014; Yassien *et al.,* 2014; Jabasingh *et al.,* 2014; Dabhi *et al.,* 2014). All tested metal ions exerted inhibitory effect on cellulase production by *B. stearothermophilus*-KGKSA40.

The application in the detergent industry does not require high-purity for enzymes and generally require use of the crude or partially purified enzyme preparation. However, it is significant to obtain enzymes with higher specific activity for their kinetic characterization. Since *B. stearothermophilus*-KGKSA40 proved to be the potent cellulytic enzyme producers, they were selected for the purpose of production, purification and investigating properties of cellulase, biosynthesized by these particular strains. Fractional precipitation of enzymes was carried out firstly by ammonium sulphate since it is highly soluble in water, cheap and has no deleterious effect on structure of protein, so for all these reasons, precipitation by ammonium sulphate was selected as a first step of purification program.

In a trial to precipitate enzymes by ammonium sulphate, results revealed that, increasing the concentration of ammonium sulphate resulted in an increase in specific activity of amylase up to 80% saturation with ammonium sulphate was proved to be the best concentration for maximal specific activity for cellulase.

Concerning other three alkaline-thermostable enzymes, the purification fold increased by 17.291 times for cellulase respectively after applying Sephadex G-200 column chromatography technique. The main fraction of each peak after sephdex G-200 and the adjacent fractions were mixed together, redialysed against sucrose and allowed to pass through the second column of purification on Sephadex G-100. The overall purification steps protocol resulted in raising the purification fold to 20.48 times for cellulase.

Detergent may contain chlorine, which degrades proteins into smaller peptide chains, thereby lowering binding energies and affecting desertion from the surface. Hence enzymes used as cleaning agents should be stable in the presence of chlorine. In the present study, data showed that, amylase was stable up to 7 µl/ml for 60 min. Cellulase retaining 100% of its activity up to 7 µl /ml for 45 min. The activity was enhanced by SDS, H_2O_2, Tween 20, Tween 80 and Triton X100 (up to 10%). Also the enzyme was stable to sodium cholate and Tween 40 (up to 5%). A decreased activity was observed by sodium preborate at all applied concentrations. The study of commercial detergents on the cellulolytic enzyme stability increases its applicability in industry. Cellulase was found to be stable in the presence of Ariel and Omo, and exhibited a decreased activity with other applied commercial detergents (Sayed, 2006).

In accordance to the present results, Grgoravski de lima *et al.* (2005) found that, celluase maintained 87 and 54 % of its initial activity upon incubation in the presence of commercial detergents as Minerva® and Ariel® respectively. While the formulations presented by Omo® was not beneficial for the enzyme. The wash performing analysis of the present enzymes revealed that, it could effectively remove a variety of stains such as blood, apple, chocolate, mango, strawberry, salad, and pomegranate while treated at 30 and 55ºC for 15 minutes by adding thermostable crude/purified enzymes separately or in combination with or without detergent.

Table.1 Relation of different incubation temperatures and pH to cellulase productivities

Incubation temperature (˚C)	**Cellulase production (unit/ml)**	**Initial pH value**	**Cellulase production (unit/ml)**
10	0.0	3	0.0
20	0.026± 0.0	5	0.008 ± 0.1
25	0.027±0.0	6	0.016 ± 0.0
30	0.028± 0.1	6.5	0.020±0.01
35	0.030 ± 0.5	7	0.038 ± 0.52
40	0.029 ± 0.22	7.5	0.086 ± 0.0
45	0.028± 0.2	8	0.1276 ± 0.0
50	0.039 ± 0.0	8.5	0.1178± 0.2
55	0.18± 0.7	9	0.21 ± 0.1
60	0.04 ± 0.2	9.5	0.1171 ± 0.0
65	0.046± 0.1	10	0.0861 ± 1.04
70	0.030± 0.132		

Table.2 Relation of different grinded palm leaf concentrations to cellulase productivity

Substrate concentration (g/flask)	**Cellulase production (U/ml)**	**Substrate Concentration (g/flask)**	**Cellulase production (U/ml)**
0.1	0.008 ± 1.04	1.5	0.0850 ± 1.5
0.2	0.0085±1.15	2.0	0.185 ± 0.0
0.5	0.016± 0.7	2.5	0.030 ± 0.52
1.0	0.068 ±2.2	5.0	0.029 ± 1.4

Table.3 Relation of application of different carbon and nitrogen sources

Carbon source	**Cellulase (s) production (U/ml)**	**Nitrogen source**	**Cellulase (s) production (U/ml)**
Control	0.1860 ± 0.52	**Control**	0.296 ± 0.0
Ribose	0.007 ± 0.0	Amm. acetate	0.046 ±1.04
D(+) Xylose	0.008 ± 0.1	Amm. molybdate	0.022 ± 0.1
D(-) Arabinose	0.1169 ± 0.0	Amm. nitrate	0.04 ± 0.0
D(-) Glucose	0.156 ± 0.2	Amm dihyd.phosphate	0.12 ± 0.52
D(+) Galactose	0.2220 ± 0.0	Amm. monohyd. phosphate	0.088 ± 0.0
D(+) Mannose	0.1168 ± 0.0	Amm. chloride	0.070 ± 0.2
D(-) Fructose	0.1866 ± 0.0	Amm. sulphate	0.321± 0.0
Rhamnose	0.007 ± 0.1	Sod. nitrate	0.065 ± 0.2
Trehalose	0.029 ± 0.0	Pot. nitrate	0.055 ± 1.5
Lactose	0.1168 ± 0.2	Mag. nitrate	0.035 ± 0.0
Maltose	0.0860 ± 0.0	Urea	0.070 ± 0.52
Sucrose	0.030 ± 0.0	Peptone	0.055 ± 0.52
Cellobiose	0.029 ± 0.0		
Raffinose	0.029 ± 0.0		
Starch	0.296 ± 0.2		
Cellulose	0.281 ± 0.52		
Dextrin	0.2200 ± 0.0		
Inuline	0.158 ± 0.2		
Mannitol	0.046 ± 0.0		

Table.4 Relation of different inocula sizes to cellulase productivity

Inocula size (ml)	Cellulase production (U/ml)	Incubation period (hours)	Cellulase production (U/ml)
0.1	0.201 ±1.5	6	0.0
0.2	0.643 ±0.2	12	0.00396 ± 0.0
0.4	0.625 ±0.52	24	0.0157±0.52
0.5	0.140 ±1.04	48	1.45 ±1.04
1	0.991 ± 0.85	72	0.3 ±0.0
1.5	2.175 ± 0.0	96	0.15 ±0.0
2	0.960 ±1.04	120	0.052 ±0.52
2.5	0.743 ±1.04		
5	1.160 ±0.52	144	UD
10	0.500 ±0.0		

Table.5 Relation of different metallic ions concentrations to cellulase productivity

Metallic ions	Concentration (ppm)	Cellulase production (U/ml)	Metallic ions	Concentration (ppm)	Cellulase production (U/ml)
Control	0.0	1.45± 0.0		1000	0.0 ± 0.0
	1000	0.04 ± 0.0		500	0.0 ± 0.0
	500	0.045 ± 0.52	$ZnCl_2$	250	0.0 ± 0.0
EDTA	250	0.035 ± 0.52		125	0.0 ± 0.0
	125	0.034 ± 0.0		50	0.0 ± 0.0
	50	0.024 ± 0.0		25	0.723 ± 0.0
	25	0.006 ± 0.0		1000	0.0 ± 0.0
	1000	0.0 ± 0.0		1000	0.0 ± 0.0
	500	0.0± 0.0		500	0.0 ± 0.0
	250	0.0 ± 0.0		250	0.045 ± 1.04
$CdCl_2$	125	0.010 ± 0.52	$CoCl_2$	125	0.052 ± 1.8
	50	0.045 ± 0.0		50	1.42 ± 1.04
	25	1.42 ± 0.6		25	1.16 ± 1.5
	1000	0.85 ± 0.7		1000	0.0 ± 0.0
	500	0.59± 0.49		500	0.007 ± 0.0
	250	0.55 ± 0.0	Sodium	250	0.0225 ± 0.0
$FeCl_3$	125	0.56 ± 1.2	selenite	125	0.046 ± 0.0
	50	0.71 ± 1.1		50	0.733± 0.0
	25	1.30± 1.9		25	0.456 ± 0.0

Table.6 Relation of different vitamin requirements application to cellulase productivity

Vitamin	Cellulase production (U/ml)	Vitamin	Cellulase production (U/ml)
Control	1.91± 0.0	Pyridoxin	0.99± 0.1
Ascorbic acid	0.520± 0.1	Riboflavin	0.75± 0.0
Nicotinic acid	0.525 ± 1.02	Folic acid	0.60 ± 0.52
Thiamine (B_1)	1.178 ± 0.5		

Table.7 Relation of different amino acids application to cellulase productivity

Amino acid	Cellulase production (U/ml)	Amino acid	Cellulase production (U/ml)
Control	1.55 ± 0.0	L–Cysteine	0.398±0.0
Glycine	1.86 ±1.00		
DL–Alanine	0.75 ± 0.0	L –Methionine	0.88±0.0
DL–Valine	0.5 ±0.52		
L–Leucine	0.77 ± 0.0	DL– Aspartic acid	3.565±0.0
Dl-Isoleucine	1.5 ± 0.28	L -Glutamine	0.399±0.0
DL-Serine	0.28±1.5	L –Arginine	0.74±0.0
DL-Threonine	0.99 ±0.2	L –Lysine	1.42± 0.0
DL–Tyrosine	1.89±1.04	L-Histidine	0.745±0.0
L–Phenylalanine	1.5 ±0.1	L–Proline	2.888±1.5
Tryptophan	0.97± 0.28		

Table.8 Relation of shaking and static conditions to cellulase productivity

(Shaking / Static) condition	Cellulase production (U/ml)
Static	2.22 ±0.0
Shaking	0.577± 1.4

Table.9 Relation of different bottle capacities to cellulose

Bottle capacity (ml)	Cellulase production (U/ml)	Bottle capacity (ml)	Cellulase production (U/ml)
100	0.554±0.0	1000	1.78±0.38
250	0.888±0.52	2000	2.5 ±0.0
500	3.9±1.5		

Table.10 A summary of the optimal nutritional and environmental parameters controlling cellulase productivity by *B. stearothermophilus*-KGKSA40 under solid state fermentation conditions

No.	Parameters	Results	No.	Parameters	Results
1	Temperature (ºC)	55	7	Incubation period(hours)	48
2	pH value	9	8	Metallic ions(ppm)	Control
3	Substrata concentration	2	9	Vitamins (100 ppm)	Control
4	Carbon source	Starch& cellulose	10	Amino acids	DL-Aspartic acid
5	Nitrogen source	Amm.sulphate	11	Shaking / static conditions	Static
6	Inoculum size (ml)	1.5	12	Bottle capacity (ml)	500

Table.11 A summary of the purification steps of cellulase produced by *B. stearothermophilus*-KGKSA40 allowed to grow on grinded Palm leaf substrate at 55 ºC under solid state fermentation conditions

Purification step	Volume (ml)	Protein content (mg/ml)	Total protein (mg)	Cellulase activity (U/ml)	Total activity	Specific activity (U/mg^{-1} protein)	Purific-ation fold	Yield (%)
Cell free filtrate	500	0.50	250	0.99	450	1.8	1	100
$(NH4)_2SO_4$ precipitation	100	1.53	153	3.350	335	2.18	2.725	74.44
Dialysis against sucrose	4.0	1.87	7.48	10	40	5.347	2.970	8.88
Sephadex G -200	5.0	0.0 24	0.12	0.75	3.75	31.125	17.291	0.83
Sephadex G -100	5.0	0.026	0.13	0.159	4.795	36.88	20.48	1.06

Table.12 A summary of amino acids analytical data of purified cellulose

No	R.T.	Amino acid	(µg/ml)	No	R.T.	Amino acid	(µg/ml)
1	11.45	Aspartic	400.741	8	32.21	Methionine	33.875
2	14.73	Threionine	198.709	9	33.85	Isoleucine	150.530
3	16.16	Serine	225.677	10	35.09	Leucine	225.660
4	**18.39**	Glutamic	515.944	11	41.98	Phenylalanine	166.155
5	25.08	Glycine	233.353	12	49.67	Histidine	310.390
6	26.36	Alanine	265.506	13	52.60	Lysine	144.020
7	29.90	Valine	169.902	14	58.79	Arginine	210.998

R.T.: Retention time

Table.13 Different Incubation temperature for maximum cellulase activity produced by *B. stearothermophilus*

Incubation temperature (ºC)	Cellulase activity (U/ml)	Incubation temperature (ºC)	Cellulase activity (U/ml)
10	0.025±0.0	60	0.530± 0.11
20	0.135±0.0	65	0.529±0.0
30	0.1560 ± 0.52	70	0.222±0.0
35	0.1560 ±0.22	75	0.155±0.0
40	0.297 ± 0.52	80	0.083 ± 0.52
50	**0.77 ± 0.22**	**85**	0.083± 0.52
55	**0.77 ± 0.52**	90-100	ND

Table.14 Thermal stability of the purified amylase

Temperature (ºC)	Cellulase activity (U/ml)	Temperature (ºC)	Cellulase activity (U/ml)
50	**0.777 ±0.0**	**75**	0.0115±0.52
55	**0.777 ±0.52**	**80**	0.0067±0.11
60	0.530± 0.0	85	0.0067±1.04
65	0.520 ± 0.0	90	0.0026±0.52
70	0.0333± 0.2		

Table.15 Different pH values in relation to the activity of the purified cellulase

pH value	Cellulase activity (U/ml)	pH value	Cellulase activity (U/ml)
6	0.3955± 0.0	9.5	0.530± 0.52
7	0.530± 0.0	10	0.521± 0.52
8	0.527± 0.0	10.5	0. 454± 0.0
9	0.755± 0.22		

Table.16 pH stability of the purified amylase and cellulose

pH value	Cellulase activity (U/ml)	pH value	Cellulase activity (U/ml)
6	0.2922±0.0	9.5	0.456±0.0
7	0.160±0.0	10	0.612±0.0
8	0.160± 0.52	10.5	0.775± 0.0
9	0.399±0.22		

Table.17 Different incubation periods in relation to the activity of the purified cellulase

Incubation period (hours)	Cellulase activity (U/ml)	Incubation period (hours)	Cellulase activity (U/ml)
1	0.0089± 0.22	15	0.223± 0.52
3	0.0460± 0.0	18	0.528± 0.0
6	0.0630± 0.0	21	0.527± 0.0
9	0.088± 0.52	24	0.755± 0.52
12	0.1188± 0.0	**36**	**1.91± 0.0**

Table.18 Different concentrations of the purified cellulase.

Enzyme concentration(µl)	Cellulase activity (U/ml)	Enzyme concentration(µl)	Cellulase activity (U/ml)
25	0.075± 0.52	125	0.466± 0.52
50	0.1175± 0.52	150	0.540± 0.0
75	0.2878± 0.0	175	0.99± 0.52
100	0.460± 0.0	**200**	**1.51± 0.0**

Table.19 Different substrate (cellulose) concentrations in relation to the activity of the purified cellulase

Substrate concentration (%)	Cellulase activity (U/ml)	Substrate concentration (%)	Cellulase activity (U/ml)
0.1	0.41± 0.0	1.0	0.61± 0.0
0.2	0.75± 0.0	**2.5**	**0.61± 0.0**
0.5	**0.61± 0.0**	5.0	0.42± 0.0

Table.20 Different chlorine concentrations in relation to the activity of the purified cellulose

Exposure time (min)	Chlorine concentration (µl/ml)			
	5		7	
	Remaining activity (%)	Enzyme activity (U /ml)	Remaining activity (%)	Enzyme activity (U / ml)
0	0.530	100.0	0.550	100.0
15	0.530	100.0	0.540	100.0
30	0.530	100.0	0.530	100.0
45	0.530	100.0	0.525	100.0
60	0.530	100.0	0.293	59.20
Exposure time (min)	**Chlorine concentration (µl/ml)**			
	10		**15**	
	Remaining activity (%)	**Enzyme activity (U /ml)**	**Remaining activity (%)**	**Enzyme activity (U / ml)**
0	0.529	100.0	0.530	100.0
15	0.529	100.0	0.41	75.0
30	0.529	100.0	0.3946	75.0
45	0.529	100.0	0.3946	75.0
60	0.290	56.2	0.2960	56.2

Table.21 Compatibility of the purified cellulase with various commercial detergents

Detergent Conc. 7µ /ml (w/v or v/v).	Control activity (Detergent & Tris buffer) U/ml	Cellulase activity (U/ml)	Final activity (%)
Powdered detergent			
Ariel	0.018	0.725±0.52	130.7
Omo	0.0	0.525±0.0	100
Lang	0.0	0.1877±0.0	35.7
Persil	0.0	0.1178±0.52	25.4
Xtra	0.0	0.344±0.0	63.7
Ariel automatic	0.0	0.2518±0.0	41.18
Xtra automatic	0.0	0.2218±0.0	42.18
Liquid detergent			
General	0.0	0.395±0.52	74.9
Pril	0.0	0.448±0.0	84.6
Control		0.536±0.0	100

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