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Application of synergistic phenomena for enhanced production of alpha 1,4 D- glucan glucanohydrolase using submerged fermentation

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ABSTRACT

The study revealed enhanced production of α 1, 4 D glucan glucanohydrolase utilizing the synergistic phenomena of bacterial hetero-culture. For this purpose, 101 hetero-cultures were screened qualitatively and quantitatively. The bacterial hetero-culture showing the highest amylolytic potential was identified as *Bacillus subtilis* and *Bacillus amyloliquefeciens* by 16S rDNA sequencing technique. Different fermentation media were evaluated and M 5 gave maximum GGH production. Different physicochemical parameters like incubation time, temperature, initial pH and inoculum size was optimized. The optimal enzyme production was obtained at 24 h, 37oC, pH 7.0 and 3% inoculum size. Glucose (3%), ammonium sulphate (1.5%) and yeast extract (2.0%) was selected as best carbon and nitrogen source, respectively. The novelty of the present piece of research was the application of the hetero-culture technique for enhanced GGH production using submerged fermentation which was not experienced before with these strains

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Introduction

Alpha-1, 4 glucan glucanohydrolase (GGH) is an extracellular enzyme that randomly splits the 1, 4- α- glyosidic linkage among adjoining glucose units inside the linear amylose and branched amylopectin chain of starch molecules and eventually produces maltose, glucose, dextrin and maltotriose (1). Alpha 1, 4 glucan glucanohydrolase holds the highest share of enzyme trades with its foremost application and it accounts for approximately 30% of the world production of an enzyme (2). GGH is a highly demanded industrial enzyme in several sectors, such as starch liquefaction, food, textile, baking, detergent and drugs. Alpha 1, 4 glucan glucanohydrolase has been derived from plants, animals and microbes. Extraction of the enzyme from animals and plants requires a bulk amount of plant material because the concentration of the enzyme is generally low and it cannot meet the demands of the market (3). However, applications for enzymes from bacterial and fungal sources have predominated in the industrial sectors. Bacterial species are the leading choice of interest over fungal species due to less time for growth, ease of process modification and optimization (4). The GGH is derived from different species of bacteria, including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *B. licheniformis*, *B.gavealus* and *B. coagulance* (5).

The cultivation procedure has a great influence on GGH production. Broad spectrum applications of the enzyme have emerged the need to search for a better fermentation process for maximum production. Submerged (SmF) and solid-state fermentation (SSF) have been explored for the production of GGH (6). Submerged fermentation is preferred because it has many advantages over solid-state fermentation (SSF), such as extraction of the product is convenient and simple to handle, and control of different parameters like temperature, pH, aeration and oxygen transfer is convenient (7,8).

Hetero-culture is a cell farming technique in which two or more dissimilar inhabitants of cells are grown with some degree of interaction among them. Hetero-culture plays a vital role in studying natural interactions among populations and enhancing cultivation success for a certain population in order to increase enzyme production (9). Hetero-culture may compose of two known or unknown bacterial species. Hetero-culture increases the growth rate. One organism produces growth factors or compounds, such as carbon or nitrogen, which are beneficial to another microorganism. The pH of the medium is altered, which is helpful for improving the activity of the enzyme. Hetero-culture permits the better utilization of the substrate. It can produce necessary nutrients for maximum performance. Hetero-culture can increase the production of GGH. Optimization of the cultural conditions has countless importance for the highest production of enzyme. It depends on the carbon and nitrogen sources, type of nutrients available to the organism, pH and incubation temperature of the medium and inoculum size (10). GGH is a key and indispensable enzyme that plays a pivotal role in the field of biotechnology. It is generally produced from microbial sources and is utilized in various industries. Industrial sectors with top-down and bottom-up approaches are now concentrating on the improvement in the GGH production

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levels by implementing different tactics. The utilization of the synergetic phenomena will hasten its industrial production. Therefore, this research was initiated with the aim to produce α 1, 4 D glucan glucanohydrolase employing the synergistic phenomena of bacterial hetero-culture. This had never been experienced before with these strains for the production of GGH in submerged fermentation. Furthermore, the study aimed to obtain the maximum production of enzymes by optimizing different process variables having a major role in the determination of the growth of strain and, subsequently enzyme production.

Materials and Methods

Isolation of organisms

The isolation of different bacterial strains was carried out from the soil samples gathered from the different locations of Pakistan by serial dilution method (11). The starch hydrolysis zone was used as the basis for initial screening. A compatibility test was carried out to test whether the two strains were compatible. Those strains were selected which did not inhibit the growth of each other. All the compatible hetero -cultures were tested for GGH production. Bacterial colonies were analyzed on the basis of morphological characteristics such as colony shape, texture, color, margins, surface characteristics, consistency and on the basis of biochemical testing according to Bergey's manual of systematic bacteriology (12). The hetero-culture showing the highest potential of GGH production was further confirmed by 16S rDNA analysis (13).

Isolation of genomic DNA

Fresh bacterial culture was used for the isolation of DNA. The bacterial cells were centrifuged at 6500 rpm (4oC) for 5 min. The pellet formed was washed using 2 ml of TEN buffer. After that, the pellet was resuspended in 1 ml of SET buffer and 100 µl of lysozyme. The mixture was incubated at 37oC for 30 min. After this 0.5 ml of TEN buffer and 50 µl of 25% SDS was added and incubated at 60oC for 15 min. 100 µl of 5M NaCl was added to the solution. The solution was treated with an equal volume of phenol: chloroform (1:1). Mixed and centrifuged at 6500 for 5 min. The upper (transparent) layer was treated with chloroform and centrifuged. The DNA was precipitated by adding a double volume of ice-chilled absolute ethanol. Spooled out the DNA and rinsed with 70% ethanol, dried and dissolved in TE buffer. Isolated genomic DNA was run in 1% agarose gel and visualized under the Gel doc imaging system. The 16s rRNA region was amplified by using the forward primer (5′-AAACTYAAAKGAATTGACGG-3′) and Reverse primer (5′-ACGGGCGGTGTGTRC-3′) under optimized PCR conditions. PCR product was run on 1% agarose gel and then observed under the Gel doc imaging system. Sequencing was carried out commercially. The PCR product, after gene cleaning, was sent to the Centre of Excellence in Molecular Biology (CEMB) for sequencing.

Use of bioinformatics tools

The sequence was analyzed with the help of BLAST and MEGA7 was used to construct a phylogenetic tree (14).

Vegetative inoculum

A loop full of selected bacterial cultures was added to sterilize nutrient broth in separate flasks. The flasks were kept at 160 rpm at 37oC in a shaking incubator for 24 h.

Fermentation media

The fermentation media having varying compositions (g/l) were assessed for GGH production. All the media were selected on the basis of the presence of starch in order to find out the GGH production ability of hetero-culture

M1

Starch 1.0 g, MgSO4.7H2O 0.5 g, KCl 0.5 g, distilled H2O 1000 ml; M2: Starch 10.0 g, Tryptone 3.0 g, Yeast extract 3.0 g, (NH4)2SO4 3.0 g, NaCl 1.0 g, Mg.SO4.7H2O 0.2 g, K2HPO4 1.0 g, Distilled H2O 1000 ml; M3: Starch 10.0 g, Peptone 5.0 g, Yeast extract 3.0 g, (NH4)2SO4 3.0 g, K2HPO4 1.0 NaCl 1.0 g, Mg.SO4.7H2O 0.2 g, Distilled Water 1000 ml; M4: Starch 10.0 g, peptone 5.0 g, Yeast extract 2.0 g, Mg.SO4.7H2O 0.5 g, NaCl 0.5 g, CaCl2 0.15 g, Distilled water, 1000 ml; M5: Starch 15.0 g, Peptone 5.0 g, Yeast extract 1.0 g, Mg.SO4.7H2O 0.5, NaCl 0.5 g, CaCl2 0.002 g, Distilled Water 1000 ml.

Submerged fermentation

1 ml of vegetative inoculum (0.5ml of each of two compatible strains) was added in sterilized fermentation medium (50ml) in all Erlenmeyer flasks (250 ml). The flasks were placed in a rotary shaking incubator at 37oC. After the incubation fermented broth was centrifuged at 6500 rpm for 15 min. The supernatant was used for the determination of enzyme activity.

Enzyme assay

The estimation of GGH was accomplished by following the method of Haq et al. (15). One ml of the crude enzyme was poured into 1.0 ml of 1% soluble starch solution. The enzyme-substrate complex was incubated at 40oC for 10 minutes. A blank was also run parallel. Reducing sugar was measured following the method of Miller (16).

The single unit activity of the enzyme is expressed as "The quantity of enzyme used to liberate reducing sugar from 1% soluble starch according to 1mg of maltose in 10min under standard assay condition."

Total protein estimation

Protein estimation was carried out following the Stoscheck (17).

Dry cell mass

Dry cell mass was find out according to Li and Mira de Orduña (18).

Statistical analysis

All the experimental data was tabulated. Post Hoc multiple comparison test under one way ANOVA and Duncun multiple range test was performed using SPSS version 20. Significance has been presented at the level of ≤ 0.05.

Results

Isolation identification and screening of bacterial hetero-culture

In the current research, different bacterial strains were

isolated from varying soil samples and identified according to Bergey's manual of systematic bacteriology (12). Primary Screening was carried out on the basis of the starch hydrolysis zone. A compatibility test was performed for all bacterial isolates. 101 compatible hetero-cultures were screened out for their enzyme production ability (Table SI& S2). Among all hetero- cultures, the hetero- culture LCWUB-.100 gave the highest enzyme yield (6.8U/ml/min). Hetero-culture showing the highest GGH production was further confirmed by 16SrDNA. The retrieved FASTA sequence was blasted using NCBI nBLAST tool. One strain of hetero-culture showed a similarity index with *Bacillus amyloliquefaciens* (99.99%) and the other with *Bacillus subtilis* (100%). The neighbor-joining method was used to construct phylogenetic tree of both bacteria using MEGA7 (Fig 1& 2). Both bacterial strains were Gram-positive, rod-shaped motile and endospore former.

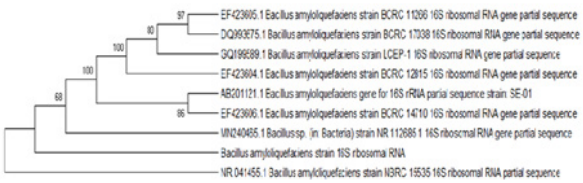

Figure 1. Phylogenetic tree of *Bacillus amyloliquefaciens* was computed using the Neighbor-Joining method conducted in MEGA7 showing close similarity to other Bacterial species

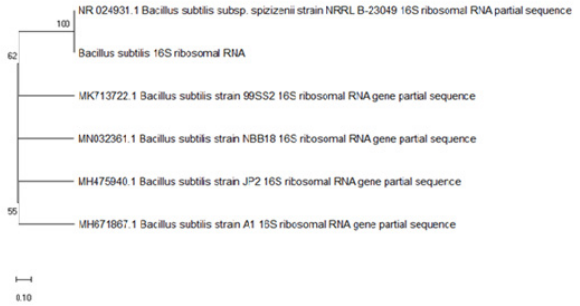

Figure 2. Phylogenetic tree of *Bacillus subtilis* was computed using the Neighbor-Joining method conducted in MEGA7 showing close similarity to other Bacterial species.

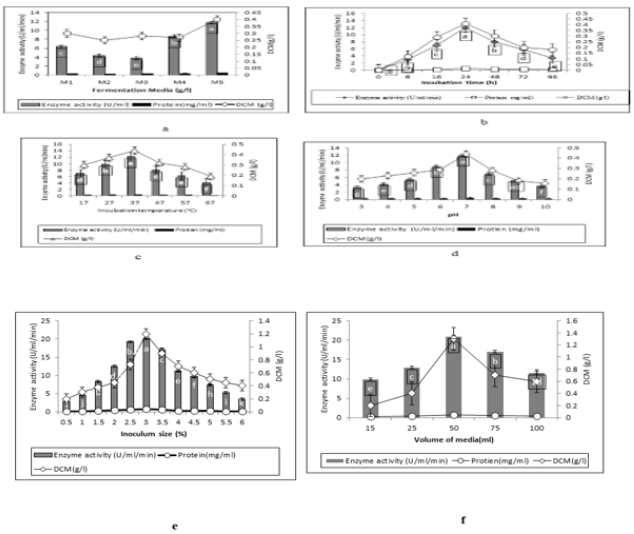

Figure 3. Effect of different environmental factors on the production of alpha 1, 4 D glucan glucanohydrolase by bacterial hetero-culture (a) Fermentation media (b) incubation time (c) incubation temperature (d) pH (e) inoculum size (f) volume of media. Each value is the mean of triplicates. Error bars indicates standard error among the replicates. Different superscripts represent that the mean difference is significant at p≤0.05 by Duncan Multiple Range test.

Screening of fermentation media

The five dissimilar fermentation media were tested for the production of GGH by bacterial hetero-culture. M5 medium (Fig3a) gave the highest production of GGH. The protein and dry cell mass were 0.47mg/ml and 0.40 g/l, respectively (Fig 3a).

Impact of Incubation time

Fermentation was conducted for 96h and GGH production was noted after every eight hours. The enzyme production was raised with the rise in incubation time. At 24 h maximum production of GGH was obtained (Fig 3b). At this time, dry cell mass and protein were 0.49 mg/ml and 0.41g/l.

Impact of incubation temperature

Fermentation was conducted at different temperatures (17oC - 67oC). Production was increased when the temperature was raised. At 37 oC maximum yield of GGH was produced (Fig 3c). At this temperature, protein was 0.42 mg/ml and dry cell mass 0.44 g/l.

Impact of varying initial pH

The influence of varying initial pH (4-11) of fermentation medium on GGH production was tested. The enzyme production was highest at pH 7.0 (Fig 3d). At this pH, protein and dry cell mass were 0.42 mg/ml and 0.44g/l, respectively.

Influence of various inoculum size

Different inoculum size (0.5-6.0%) for maximum enzyme biosynthesis was investigated (Fig3e). The inoculum size of 3% gave the maximum enzyme production. At 3% inoculum size, dry cell mass and protein were 1.2g/l and 0.72mg/ml, respectively.

Impact of different volume of medium

Different volume of fermentation medium (15, 25, 50, 75, 100 ml) for GGH production was tested. 50 ml of fermentation medium was found to be the best because, at this volume, maximal GGH was produced (Fig 3f). The total soluble protein was 0.73 mg/ml and the dry cell mass was1.30 g/l at this point.

Influence of additional carbon sources

The impact of varying carbon sources like xylose, ribose, glucose, fructose, maltose, glycerol and carboxy methyl cellulose (CMC) was screened for GGH production (Fig 4a). From all screened carbon sources, glucose indicates a significant increase in enzyme production. So, glucose was selected as the optimized carbon source and different concentrations (0.5-4.0%) were added to the fermentation medium (Fig 4b). The glucose at the concentration of 2.5% produced the maximum enzyme. At this concentration, dry cell mass and protein were 2.6 g/l and 1.1 mg/ml, respectively. Above this concentration of glucose, there was a decrease in enzyme yield.

Optimization of nitrogen sources

In the current study, different nitrogen sources, both inorganic and organic and their concentrations were tested for optimal GGH production. The included nitrogen sources were NH4 NO3, KNO3, (NH4)2 SO4, NH4Cl2, tryptone, peptone, urea and yeast extract. The maximum enzyme production was achieved when 1.5% ammonium sulphate was added to the fermentation medium (Fig 4c& 4d). In the case of organic nitrogen sources, maximal GGH was produced in the presence of 2% yeast extract (Fig 4e & 4f). The dry cell mass was 2.9 g/l at this level.

Effect of metal ions

Different metal ions, including NaCl, MgSO4, CuSO4, FeSO4, CaCl2, MnCl2, and ZnSO4, were added to the fermentation medium. NaCl and MgSO4 had inhibitory effects of all metal ions, while others showed a stimulatory effect. CuSO4 had the maximum stimulatory effect on enzyme production. There was a considerable increase in enzyme production by its addition in the fermentation medium (Fig 5).

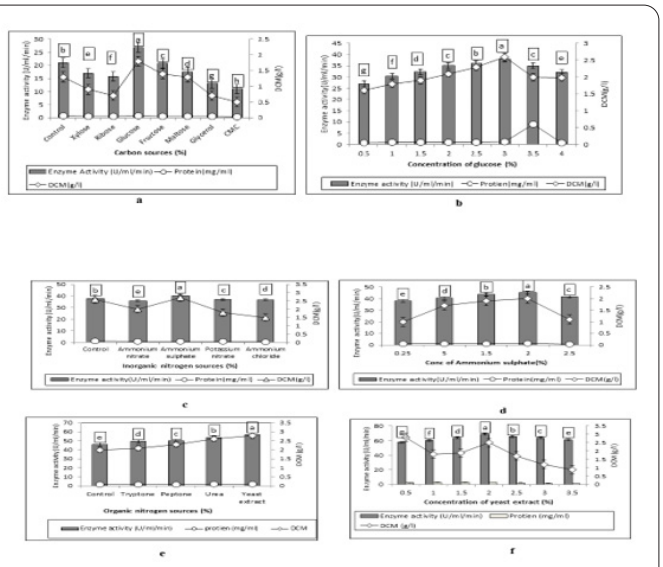

Figure 4. Impact of nutritional factors on the production of alpha 1, 4 D glucan glucanohydrolase by bacterial hetero-culture (a) carbon sources (b) concentration of glucose (c) inorganic nitrogen sources (d) concentration of ammonium suphate (e) organic nitrogen sources (d) concentration of yeast extract. Each value is the mean of triplicates. Error bars indicates standard error among the replicates. Different superscripts represent that the mean difference is significant at p≤0.05 by Duncan Multiple Range test.

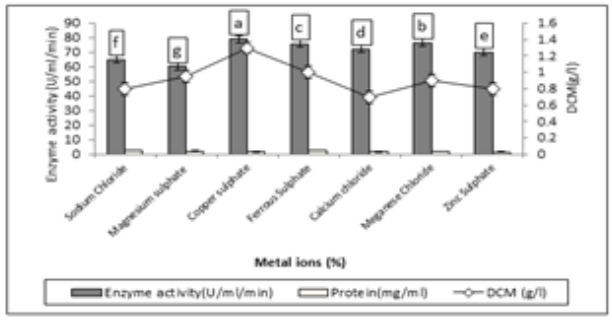

Figure 5. Influence of metal ions on the production of alpha 1, 4 D glucan glucanohydrolase by bacterial hetero-culture. Each value is the mean of triplicates. Error bars indicates standard error among the replicates. Different superscripts represent that the mean difference is significant at p≤0.05 by Duncan Multiple Range test.

Discussion

The Selection of appropriate bacterial hetero- culture and optimization of environmental and nutritional factors is vital for the successful biosynthesis of α 1, 4 D glucan glucanohydrolase by the fermentation process. In the current study, compatible bacterial hetero-cultures isolated from different soil samples were screened. Bacterial hetero-culturing increased GGH production. It might be due to the reason that two different, but compatible bacterial strains, when grown together, can enhance the growth of one another. One strain may release such chemicals or growth factors that can favour the growth of another strain (9). All the isolated bacterial strains were subjected to Gram staining for morphological identification. The result revealed that all the bacterial isolates were Gram-positive. Perhaps it might be due to the presence of a thick peptidoglycan layer in the cell wall of bacteria. They retain the blue color of crystal violet and decolorizer cannot remove the stain from the cell wall. So when the counter stain was applied and examined under the microscope, all strains were Gram-positive (19). Endospore staining is also used for the identification of bacterial strains on the basis of the presence and absence of endospores. In the present study, 87% of bacterial strains were endospore former it might be due to the reason that Genus *Bacillus* mostly dispersed in soil, and these strains were isolated from soil samples. Taxonomy of the *Bacillus* species represents its most species are endospore-forming (20). Further identification was carried out by other biochemical tests, including a motility test, catalase and oxidase test. In the current research, all the bacterial strains were catalase positive and the majority were oxidase positive. The reason might be that these bacterial strains possess catalase and oxidase enzymes. 81% of strains were motile, while the remaining were non-motile. The motility was due to the presence of flagella, while non-motile strains do not possess flagella.

Further confirmation of the bacterial strains was carried out by 16S rDNA sequencing technique. The 16S rDNA gene sequence has been considered the most accurate and convenient for identifying microorganisms (21). It is present in all bacteria, and the purpose of the 16SrDNA gene over time has not been altered. It is a conserved region so identification is appropriate by this technique (22). In the current study, the heteroculture that possess the highest amylolytic potential were identified as *Bacillus subtilis* and *Bacillus amyloliquefeciens*. Both bacterial strains were Gram-positive, rod-shaped motile and endospore former (23).

Fermentation media play a critical part in the production of enzymes by bacterial hetero-culture. It supplies the important nutrients which are important for bacterial growth as well as enzyme secretion (24). In the current study, the M5 medium was found to be the best for maximum enzyme production. Probably the reason was that peptone and yeast extract are organic nitrogen sources containing free amino acids, which enhance enzyme production in addition to this M5 medium having metal ions like Ca+2 Cl-, Mg+2 and SO-4 which are very essential for bacterial growth and sometime Ca+2act as a stimulator for enzyme production (25). All the other media gave

less production of enzymes by bacterial hetero- culture, it might be ascribable to the reason the absence of any significant element which is indispensable for the development of bacteria and consequently for enzyme production.

The incubation period is also an indispensable component for the growth of bacteria and, accordingly, enzyme production. In the present research, fermentation was conducted for 96h. At 24 h maximum production of GGH was obtained. The reason might be that bacteria entered into a stationary phase after 24h of fermentation. During this period, there was a gradual decrease in enzyme biosynthesis. This decline was due to the reason that crucial nutrients mandatory for bacterial growth were depleted, which caused the inactivation of the secretory machinery of the enzyme by Bacillus (26), or due to the accumulation of other by-products (27). The present work was more encouraging than the work of Deb et al. (28), who reported the optimum time for enzyme production was 72 h, while at present, its 24h. So a decrease in the period saved energy and made the process more economical. The incubation temperature plays pivotal in enzyme production. The maximum GGH production was obtained at 37oC. Above this temperature, there was a reduction in enzyme yield. The cause might be that high temperature has a deleterious effect on bacterial growth, so as the bacterial hetero- culture growth was suppressed, there was a decline in enzyme production, too (29, 30). Amongst the physiochemical parameters, the pH of the fermentation medium has a key role in enzyme production by inducing morphological variation in bacterial growth. In the present research, bacterial hetero-culture produce optimal GGH at pH 7.0. Beyond this level, there was a gradual decrease in GGH production. The reason might be that the production of GGH is very sensitive to H+ ion concentration (31). Our findings are according to Hasan et al. (32) who reported that neutral pH is optimum for bacterial growth, so when bacterial growth is increased enzyme production also enhanced.

The size of the inoculum has a positive impact on the growth of organisms and enzyme production. In the present study, 3% inoculum size produced the maximum enzyme activity. Reducing enzyme production at higher inoculum concentrations might be ascribable to the declined nutrient availability for bacterial growth, so when bacterial growth inhibits enzyme production as well. When inoculum size was small from the optimum level, there was also a decline in enzyme production. Possibly it was due to the inadequate growth of bacterial hetero-culture to a produced enzyme or other toxic metabolites (33). Optimization of the volume of media is also very essential for nutrient supply, agitation, microorganism growth and production of the enzyme. In the current research, the highest enzyme production was attained in 50 ml of fermentation medium. However, with the increase in the volume of medium reduction in GGH production occurred. It might be due to the reason that the reduction in the agitation rate of medium, decreased air supply ultimately reduces enzyme biosynthesis. At a low volume of fermentation medium, enzyme production was also reduced. It might be due to the non-availability of adequate nutrients required for the growth of bacterial hetero- culture (34).

Different carbon sources are important for bacterial growth and enzyme biosynthesis (35). Production of the alpha 1, 4 D glucan glucanohydrolase and cell growth is also influenced by carbon sources in the shake flask (36). A remarkable enhancement in enzyme production was recorded in the presence of glucose. Perhaps it was due to the cause that glucose was metabolized easily by hetero- culture. So as the bacterial growth was enhanced, there was an increase in enzyme production. Upon addition of 3% of glucose in the fermentation medium maximum GGH production was achieved. Further upsurge in the amount of glucose reduced the enzyme yield. Perhaps it was due to the cause that lesser amount of carbon was inadequate for the development as well as enzyme biosynthesis; while extra carbon was equally disadvantageous and cause catabolic suppression (37). Nitrogen sources play a key role in enzyme production as well as organism growth. In the current study, different inorganic and organic nitrogen sources were tested for the biosynthesis of GGH by bacterial hetero-culture. The maximum enzyme production was obtained when (NH4)2SO4 was added to the fermentation medium. It might be that ammonium sulphate provided both ammonium and sulphate ions for cell growth as well as for enzyme production. 1.5% ammonium sulphate gave the highest enzyme production; further increase in concentration reduces the yield of alpha 1, 4 D glucan glucanohydrolase by bacterial hetero-culture. It might be that a high concentration of inorganic nitrogen has an inhibitory effect on the production of GGH. Out of all tested organic nitrogen sources, yeast extract, along with ammonium sulphate gave the maximum enzyme production. Yeast extract at the level of 2% produces optimal GGH. Beyond this level, there was a decrease in enzyme production. Our findings were in accordance with the work reported by Gervais & Molin (38), who described that high or low levels of yeast extract from optimal value caused a decline in enzyme production.

The optimum concentration of metal ions is very crucial for enzyme production, as these act as a stimulator or an inhibitor in enzyme production (39). GGH is a metalloenzyme. In this study, different metal ions were tested. These include NaCl, MgSO4, CuSO4, FeSO4, CaCl2, MnCl2 and ZnSO4. Among all of these, CuSO4 gave the maximum enzyme production. The cause might be that CuSO4 act as a stimulator and enhance the production of GGH (40). NaCl and MgSO4 acted as an inhibitor because these inhibited the production of GGH. The cause might be that these metals block the binding sites of enzymes (41).

In the present study, alpha 1, 4 D glucan glucanohydrolase was produced by bacterial hetero-culture using submerged fermentation. The utilization of novel techniques of bacterial hetero-culturing and optimization of cultural conditions reduce the cost of GGH production.

Conflict of interest

No conflict of interest associated with this work.

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