Random mutagenesis and process optimization of bacterial co-culture for hyperproduction of 1, 4-α-D-glucan glucanohydrolase using submerged fermentation

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Abstract

The exponential increase in the application of 1,4-α-D-glucan glucanohydrolase (GGH) in various fields has placed stress and demand in both qualitative improvement and quantitative enhancement through strain improvement. In the present work, *Bacillus subtilis* LCBT-15 and *Bacillus amyloliquefaciens* LCBT-20 were subjected to physical as well as chemical mutagenesis for improving the GGH production potential. Applications of the UV light and ethidium bromide did not cause a significant increase in the enzyme production. However, Ethyl methane sulphonate (EMS) treated co-culture 10 gave 1.3-fold increase in the GGH production, in contrast to the wild co-culture. Different physicochemical parameters including fermentation media, rate of fermentation, temperature, pH, nitrogen and carbon sources and surfactants were also investigated. The M7 medium composition was optimized for GGH production after 48h of incubation at 37°C and pH 6. The optimum inoculum size was 3.5 ml (1×10^6 cells/ml) in 50 ml of medium. The best carbon and nitrogen sources were lactose (2.5 %); ammonium chloride (1.75 %) and beef extract (1 %), respectively. Optimal GGH production (287 U/ml) was obtained when the medium was supplemented with 0.05% Tween 80. The novelty of this work was exploration of the synergistic phenomena of mutant bacterial co-culture for the enhancement of GGH production.

Keywords: *mutation; ethyle methane sulphonate; co-culture; 1, 4-α-D-glucanglucanohydrolase; Bacillus*

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1. INTRODUCTION

1,4-α-D-glucan glucanohydrolase (EC 3.2.1.1; GGH) is an extracellular enzyme produced by a variety of microorganisms. This enzyme randomly cleaves the α-1,4 linkages between adjoining glucose units in a starch molecule ultimately producing oligosaccharides of varying lengths such as maltotriose, maltose and glucose [1-2]. GGH is very important for industrial purposes and constitutes approximately 25 % of the enzyme market. GGH is used in textile, sugar, pharmaceutical, chocolate, bread and chapatti, alcohol, paper, detergent, building products and feed industries [3-4].

1,4-α-D-glucan glucanohydrolase can be obtained from plants, animals and microorganisms. For industrial production of this enzyme, microorganisms (fungi and bacteria) are preferred, more than plants and animals, due to their advantages such as simple manipulation, ease of cultivation, cost effectiveness, shorter enzyme production time and easier process of modification. Among bacteria, genus *Bacillus* is widely used for the industrial production of GGH and preferred over fungi due to its rapid growth, short fermentation cycle and safe handling [4].

Co-culture is a cell cultivation technique by which cells of two or more different populations grow with some level of connection between them. Co-culture plays an important role to study natural interactions between populations for the enhanced production of enzymes [5]. A co-culture may be consisted of two known or unknown species. This technique has many advantages over the single culture technique such as high product yield, better consumption of substrate and prevention of contamination. In addition, due to production of growth factors or compounds by one microorganism, which are beneficial for the other microorganism, the growth rate in the co-culture increases [6].

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Strain improvement plays a vital role in the enhancement of many microbial fermentation processes. The improved strain with special characteristics can decrease the production cost while increasing the production of desired products. The strain improvement can be attained by introducing genetic changes in the wild strain [7]. Hence, a major effort in industrial research, in producing enzymes, is directed towards strain improvement and screening programs. Successful mutant strains can be obtained by using conventional mutagenesis techniques such as ultraviolet (UV) radiation and treatment with chemical mutagens like ethidium bromide, ethyl methane sulphonate (EMS), N-methyl-N'-nitro-N-nitrosoguanidine (NG) and nitrous acid. In the process of strain improvement, microorganisms are subjected to the above mentioned mutagens and subsequently appropriate selection and screening are carried out [8]. Mutation by physical and chemical mutagens is random and occurs at any time. It should be mentioned that there are some other methods of mutation like recombinant DNA technology, but random mutagenesis is more beneficial due to its economical procedure and simplicity [9].

Improvement of system performance to raise the enzyme yield without increasing the production cost is very important to fulfill industrial demands. Physico-chemical conditions such as pH, temperature, size of inoculum and incubation time, as well as media components and their concentrations, play a vital role for optimal enzyme production. Therefore, the optimization of chemical and physical parameters is crucial because it has a large effect on the feasibility and economy of the fermentation process [10-12]. The bacterial co-culturing and utilization of synergetic phenomena enhance the production of GGH both in solid and submerged fermentation. The GGH production by bacterial co-culture of (*Bacillus cereus and Bacillus thuringenesis*) was increased as a result of optimization in solid state fermentation [13]. The aim of present study was to enhance the GGH production by improving the wild co-culture by induction of a mutation in the gene sequence.

2. MATERIALS AND METHODS

2. 1. Materials

All the chemical used in this studied were of analytical grades: ethyl methane sulphonate (Sigma USA), nutrient broth (Merck Germany), ethidium bromide (Merck Germany), 2 deoxy-D- glucose (Sigma USA), yeast extract (Merck Germany), magnesium sulphate (Sigma USA), peptone (Fluka Switzerland), calcium chloride (Merck Germany), ammonium sulphate (Sigma USA), tryptone (Sigma USA), glucose, lactose, fructose, maltose (Merck Germany), starch soluble (Merck Germany), sodium chloride (Merck Germany), sodium hydroxide (Sigma USA), HCl (Sigma USA), Comaisee Brilliant Blue G-250 (Sigma USA), and beef extract (Sigma USA).

2. 2. Organism

Bacillus subtilis LCBT-15 and *Bacillus amyloliquefaciens* LCBT-20 were obtained from the biotechnology laboratory of Lahore College for Women University (Lahore, Pakistan).

2.3. Preparation of the bacterial suspension/inoculum

A loop full of bacterial culture was transferred in 50 ml of sterilized nutrient broth. After inoculation flasks were kept in a shaking incubator at 37 °C for 24 hours.

2. 4. Random mutagenesis

2. 4. 1. UV irradiation (Physical Mutagenesis)

Physical mutagenesis was carried out by UV irradiation (dose 1.6×10^2 J/m^2). For this purpose, 5 ml of bacterial suspension was transferred in a sterile Petri plate and exposed to UV light for 30 - 120 min. The distance of the Petri plate from the UV lamp was 8 cm [14].

2. 4. 2. Chemical mutagenesis

Chemical mutagenesis was carried out by treating the bacterial suspension with analytical grade ethidium bromide and EMS (Merck, Germany).

2. 4. 2. 1. Ethidium bromide treatment

2 ml of bacterial suspension was treated with 50 µl 1 % ethidium bromide (30 – 120 min). After a specific time interval, the treated suspension was centrifuged at 6000 rpm for 15 min. Supernatant was discarded and the pellet was washed thrice with phosphate buffer to elute the ethidium bromide. After washing, the pellet was resuspended in the same buffer.

2. 4. 2. 2. Ethyl methane sulphonate treatment (EMS)

2 ml of bacterial suspension was treated with 50 µl EMS (30-120min). After the specific time interval, the suspension was centrifuged at 6000 rpm for 15 min. Supernatant was discarded and the pellet was washed thrice with phosphate buffer to elute the EMS. After, washing pellet was resuspended in the same buffer [15].

2. 4. 3. Mutant selection

The 0.1 ml aliquot of all treated bacterial samples was transferred to Czapekdox starch agar medium plates supplemented with 0.4 % (w/v) 2 deoxy-D- glucose and incubated at 37 °C for 24 hours. After incubation, plates were flooded with 2 % iodine solution and colonies showing a larger zone of starch hydrolysis in contrast to wild strains were selected and subjected to secondary screening.

2. 5. Fermentation media

Following fermentation media in g/l were evaluated for the production of GGH using submerged fermentation. **M1:** Yeast extract 1.0, soluble starch 15, $MgSO_4$ 0.5, Peptone 5, $CaCl_2$ 0.002, NaCl 0.5 [15]; **M2:** Bactopeptone 14.0, lactose 10, $MnSO_4$ 0.001, yeast extract 6, $MgCl_2$ 0.2, $CaCl_2$ 0.25, KCl 1, $FeSO_4$ 0.0005 [14]; **M3:** $MgSO_4$ 0.5, peptone 6.0, starch 1.0, KCl 0.5 [16]; **M4:** Starch 10, Tryptone 3, K_2HPO_4 1, $(NH_4)_2SO_4$ 3, NaCl 1, $MgSO_4$ 0.2, yeast extract 3 [15]; **M5:** Starch 10, peptone 5, K_2HPO_4 1, $(NH_4)_2SO_4$ 3, NaCl 1, $MgSO_4$ 0.2, yeast extract 3 [17]; **M6:** Starch 10, peptone 5, Yeast extract 2, $(NH_4)_2SO_4$ 3, $MgSO_4 \times 0.7\ H_2O$ 0.5, $CaCl_2$ 0.15 and NaCl 0.5[15]; **M7:** Starch 15, peptone 5, Yeast extract 20, Glucose 30, $(NH_4)_2SO_4$ 15, $MgSO_4 \times 0.7\ H_2O$ 0.5, $CaCl_2$ 0.15, NaCl 0.5; **M8:** Yeast extract 5, NaCl 10, Tryptone 15, Starch 10, $(NH_4)_2SO_4$ 1[18].

2. 6. Submerged fermentation

50 ml of the sterilized fermentation medium was inoculated with 0.5 ml of inoculum from each of both mutant strains. The flasks were incubated in a shaking incubator at 37 °C for 24 hours. After the precise time of incubation, the fermented broth was centrifuged at 6000 rpm for 15 minutes. The supernatant was used for enzyme estimation. All the experiments were run in triplicates.

2. 7. Influence of different parameters

The influence of different parameters including temperature, inoculum size, volume, pH, carbon and nitrogen sources etc. on the GGH production by mutant co-culture was estimated. The pH of optimized medium M7 was changed ranging from (4-10). The different pH was adjusted by using 1 M HCl and 1 N NaOH in order to evaluate the effect of pH. Different in organic and organic nitrogen sources and surfactant were added in 0.5, 0.25 and 0.02 % respectively, in fermentation medium in order to evaluate the effect these factors on the GGH production.

2. 8. Enzyme assay

Enzyme assay was executed according to Haq *et al.* [19]. 1ml of crude enzyme and 1ml of 1 % soluble starch solution were added to a test tube. The enzyme substrate mixture was incubated at 40 °C for 10 minutes. A blank was also run in parallel. Reducing sugar content was estimated according to Miller [20]. One unit of GGH activity was defined as the "amount of enzyme used to liberate reducing sugar from 1 % starch solution according to 1 mg of maltose in 10 minutes under the standard assay condition".

2. 9. Protein estimation

Protein estimation was carried out by the method of Bradford [21].

2. 10. Dry cell mass

Dry cell mass was calculated according to Li and Orduña [22].

2. 11. Statistical analysis

All the data were tabulated and statistically analyzed. Post-Hoc Multiple comparison tests were applied using one-way ANOVA by using the software package SPSS (version 22, IBM). The mean difference is significant at the level of ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3. 1. Random mutagenesis

Production of the enzyme from a wild strain is usually low. Therefore, strain improvement and optimization of culture conditions play a vital role in enhancing the enzyme production [23]. For this purpose, two *Bacillus* species *Bacillus subtilis* LCBT-15 and *Bacillus amyloliquefaciens* LCBT-20 were subjected to different mutagens such as UV light, ethidium bromide and EMS (30-120min) for better production of GGH. The current study showed that UV irradiation and ethidium bromide did not cause any significant increase in GGH production, in contrast to wild strains (data not shown). By increasing the time of treatment, the number of survivors was reduced. However, after the EMS treatment, a significant increase in GGH production was observed. Out of seventeen mutant derivatives (Table 1), the mutant co-culture of *Bacillus subtilis* EMS-10 and *Bacillus amyloliquefaciens* EMS-10 was found to be the best and used for further optimization. Probably the reason was a mutation caused by the substitution of nucleotide and alkylation of guanine.

EMS is an effective mutagen for *Bacillus* species as reported by Suribabu *et al.* [24]. A similar observation was also reported by Haq *et al.* [14] that UV light did not increase the GGH production while EMS is a potent mutagen for GGH production enhancement.

Table 1: Screening of the EMS treated co-culture of Bacillus subtilis LCBT-15 and Bacillus amyloliquefaciens LCBT-20 for GGH production using submerged fermentation

EMS treatedco-culture (50µl)	Time of exposure, min		Enzyme activity, U/ml	Total protein concentration, mg/ml	DCM, g/l
	B. subtilis LCBT-15	*B. amyloliqu-efaciens* LCBT-20			
Wild			54±0.01	0.42±0.01	8±0.01
EMS co-culture 1	0	30	35±0.1	0.27±0.03	6.7±0.01
EMS co-culture 2	0	60	57±0.5	0.45±0.05	8.3±0.01
EMS co-culture 3	0	90	59±0.2	0.48±0.01	8.3±0.2
EMS co-culture 4	0	120	59±0.01	0.48±0.01	8.4±0.1
EMS co-culture 5	30	30	54±0.01	0.42±0.01	8±0.01
EMS co-culture 6	30	60	23±0.05	0.25±0.02	2.8±0.05
EMS co-culture 7	30	90	50±0.02	0.38±0.04	7.5±0.02
EMS co-culture 8	60	60	49±0.04	0.37±0.07	7.6±0.03
EMS co-culture 9	60	30	56±0.5	0.45±0.01	8.3±0.3
EMS co-culture 10	90	30	74±0.02	0.68±0.08	11±0.05
EMS co-culture 11	90	120	43±0.05	0.30±0.01	7.0±0.5
EMS co-culture 12	120	30	47±0.01	0.36±0.06	7.4±0.3
EMS co-culture 13	120	120	59±0.07	0.48±0.05	8.3±0.01
EMS co-culture 14	30	0	56±0.06	0.43±0.03	8.2±0.2
EMS co-culture 15	60	0	57±0.01	0.46±0.03	8.5±0.1
EMS co-culture 16	90	0	54±0.08	0.40±0.01	7.9±0.05
EMS co-culture 17	120	0	11±0.04	0.16±0.02	1.7±0.02

Each value is the mean of triplicates. Difference of mean is significant at the level of $p \leq 0.05$. ± shows the standard deviation among triplicates. DCM= Dry cell Mass

3. 2. Screening of fermentation media for GGH production

Composition of medium noticeably affects the bacterial growth, which eventually affects the enzyme production. Therefore, selection of appropriate fermentation media is important for the enzyme production improvement. Eight different fermentation media (Section 2.5) were tested for the GGH production by the mutant bacterial co-culture. Among all, M7 gave the highest yield of GGH (74 U/ml). The dry cell mass and total protein content were 11 g/l and 0.68 mg/ml, respectively (Fig. 1a). This result may indicate that M7 medium contains pre-requisite nutrients that support the GGH production [25]. Metal ions such as Ca^{2+} and Mg^{2+} were shown to be essential for bacterial growth and Ca^{2+} was an activator in GGH production [26]. GGH production was lower in all other investigated media. The reason might be in the absence of some essential element or the presence of some component that inhibits the bacterial growth and enzyme production [27].

3. 2. 1. Influence of the fermentation rate on GGH production

The impact of different incubation times (24-120 hours) on the GGH production by mutant co-culture was analyzed. It was noticed that the maximum enzyme production (80 U/ml) was obtained after 48 h of incubation. Further rise in the incubation time resulted in a decline in the GGH production (Fig. 1b). This result might be due to the reduction of nutrients in the medium, enzyme denaturation and production of by products that constrain the biosynthesis of GGH by a feedback inhibition mechanism [28].

3. 2. 2. Influence of the incubating temperature on GGH production

The influence of varying incubating temperature (27 – 67 °C) on the GGH production is shown in Figure 1c. Increase in the temperature induced the increase in enzyme production, which reached a maximum (80 U/ml) at 37 °C. Below or above this temperature, production of GGH was decreased. The reason might be that at high temperatures, the growth of microorganisms is inhibited, which eventually reduced the enzyme production. On the other hand, at low temperatures the enzyme production was lower due to the decrease in the bacterial growth rate and changes in the cell composition [29].

3. 2. 3. Influence of various media volumes on the GGH production

Optimization of medium volume is also necessary for uniform nutrient supply, growth of microorganisms and enzyme production. Various volumes of media (25-150 ml) were analyzed for the production of GGH by the mutant co-culture (Fig. 1d). Among all, 50 ml medium volume was found to be the best with the elevated GGH production (80 U/ml). Further increase or decrease in the medium volume resulted in reduction of GGH production. The reason might be that above the 50 ml medium volume, the agitation rate and air supply in the medium was reduced, which subsequently reduced the enzyme production. On the other hand, at the lower medium volume, the enzyme production might be decreased due to the insufficient availability of nutrients [30].

3. 2. 4. Influence of the size of inoculum on the GGH production

The production of GGH was investigated at various inoculum sizes (0.5-4 ml), while 1 ml of inoculum contains 1×10^6 cell (in the Fig. 1e). The maximum GGH production (86 U/ml) was obtained with 3.5 ml inoculum in the fermentation medium. Above this value, there was a reduction in the GGH biosynthesis due to depletion of nutrients for the high biomass concentration and fast growth of microorganisms. On the other hand, below the optimal level, a decline in the GGH production might be due to the slower microbial growth and thus a longer time period to reach the stationary phase [31].

3. 2. 5. Influence of pH on the GGH production

The impact of various pH values (4 – 10) of the fermentation medium on the GGH production in the shake flask fermentation is presented in Figure 1f. The elevated GGH production (92 U/ml) was observed at pH 6. Above this pH, a decline in enzyme production was observed possibly due to the suppression of bacterial metabolic activity that eventually reduced the GGH production [32]. Our findings are in accordance with Mahalakshmi and Jayalakshmi [33], that have also found the elevated GGH production at pH 6.

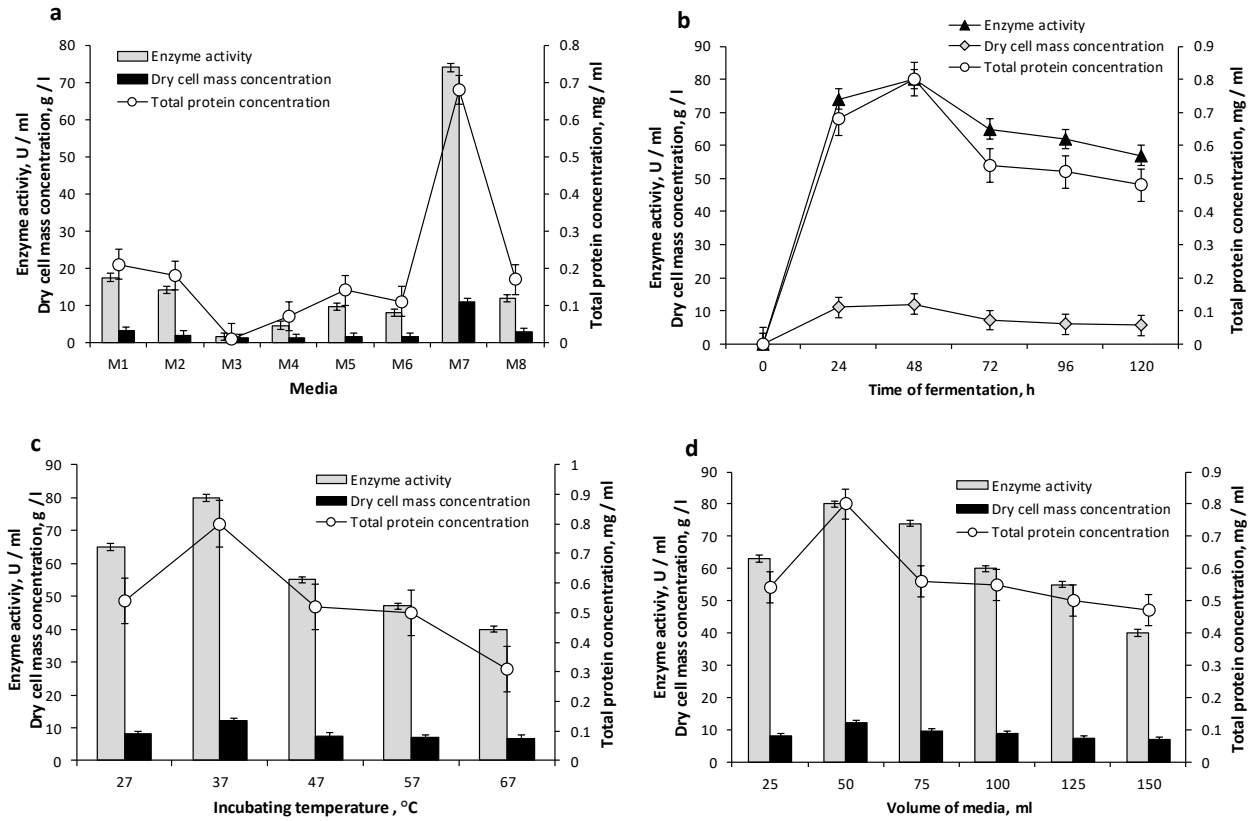

Figure 1a-d. Influence of different parameters on the production of 1, 4-á-D-glucan glucanohydrolase by mutant co-culture of Bacillus subtilis EMS-10 and Bacillus amyloliquefaciens EMS-10: (a) Fermentation media, (b) rate of fermentation, (c) incubating temperature, (d) volume of media

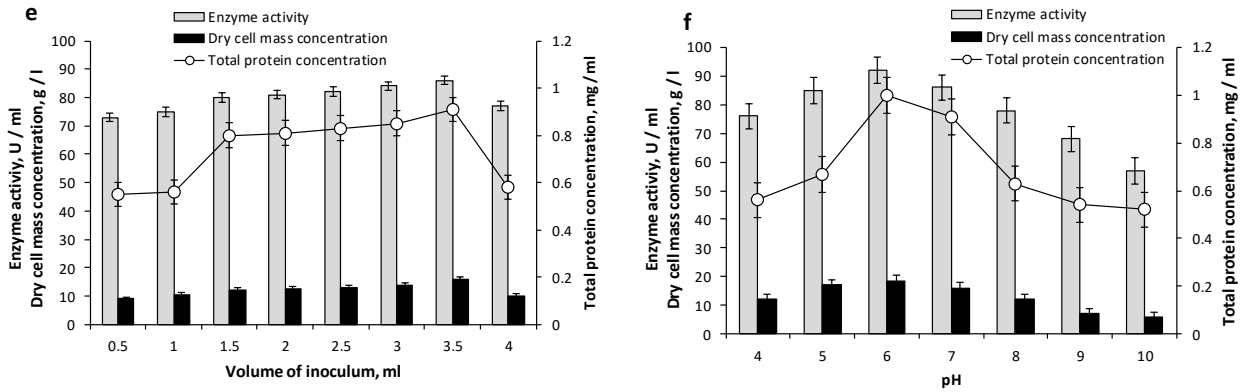

Figure 1 e-f. Influence of different parameters on the production of 1, 4-á-D-glucan glucanohydrolase by mutant co-culture of Bacillus subtilis EMS-10 and Bacillus amyloliquefaciens EMS-10: (e) volume of inoculum, (f) pH of medium;

3. 3. 1. Influence of various carbon sources on the GGH production

Influence of various supplemented carbon sources (1 %) such as lactose, glucose, fructose, maltose, molasses, starch and xylose were evaluated for the GGH production (Fig. 2a). A noticeable enhancement in the GGH production (103 U/ml) was observed when the medium was supplemented with lactose. This result correlates with that of Haq *et al.* [14], who reported the lactose as the best carbon source for GGH production. So, in the next experimental series, lactose was supplied at various concentrations in the range 0.5- 3 % (Fig. 2b). The maximal enzyme production (114 U/ml) was achieved at the lactose concentration of 2.5 %. Below or above this value; GGH production was decreased possibly due to insufficient carbon supply for bacterial growth and enzyme production at low lactose concentrations and catabolic suppression at high lactose concentrations which were harmful [34].

3. 3. 2. Influence of various inorganic nitrogen sources on GGH production

Effects of different inorganic nitrogen sources such as ammonium nitrate, ammonium chloride, sodium nitrate, potassium nitrate and ammonium sulphate on the GGH production is presented in Figure 2c. Elevated GGH production (126 U/ml) was observed when the medium was supplemented with ammonium chloride, which was then supplied at various concentrations (0.25 – 2 %; Fig. 2d). The highest GGH production (141 U/ml) was achieved at the concentration of 1.75 %. Reduction in enzyme production at higher ammonium chloride concentrations might be due to lowering of the pH value as well as protease induction that inhibited the GGH production [35]. Ammonium sulphate and sodium nitrate inhibit the GGH production, which might be due to the fact that sodium and sulphate ions caused deleterious effects on bacterial growth [36]. Similar results were also reported by Saxena *et al.* [37].

3. 3. 3. Influence of various organic nitrogen sources on the GGH production

Different organic nitrogen sources (beef extract, tryptone, peptone and urea) at the concentration of 0.25 % were assessed for the production of GGH (Fig. 2e).

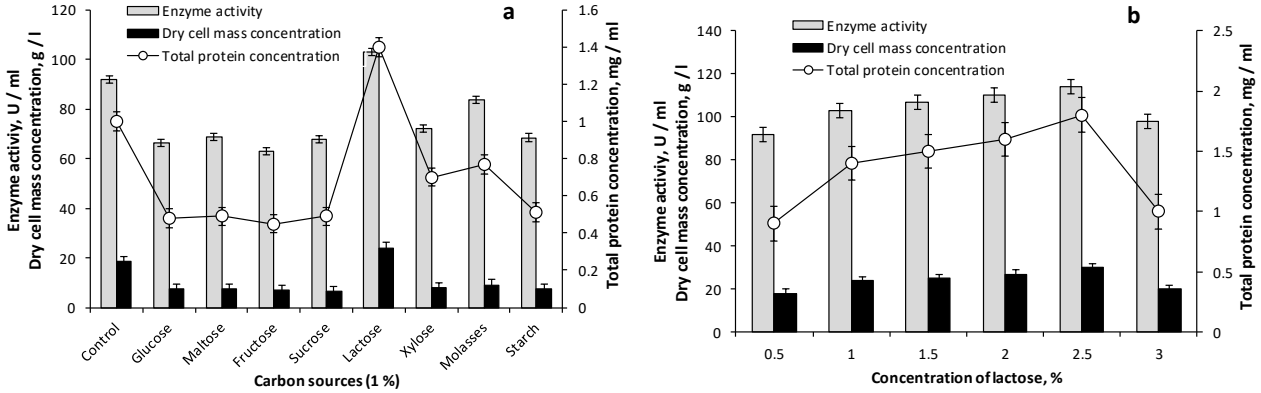

Figure 2a-b: Influence of various nutritional factors on the production of 1, 4-á-D-glucan glucanohydrolase by mutant co-culture of Bacillus subtilis EMS-10 and Bacillus amyloliquefaciens EMS-10: (a) Carbon sources, (b) concentrations of lactose

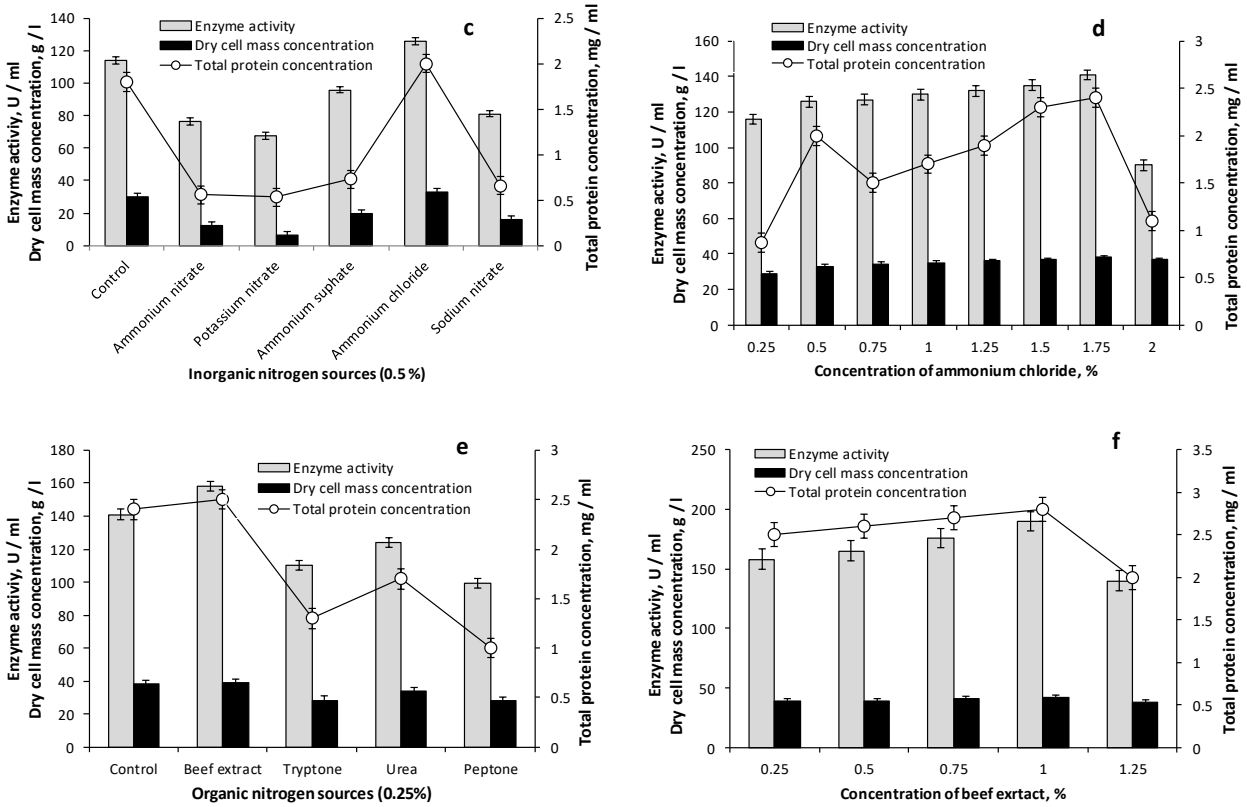

Figure 2c-d: Influence of various nutritional factors on the production of 1, 4-á-D-glucan glucanohydrolase by mutant co-culture of Bacillus subtilis EMS-10 and Bacillus amyloliquefaciens EMS-10: (c) Inorganic nitrogen sources, (d) concentrations of ammonium chloride, (e) organic nitrogen sources, (f) concentrations of beef extract

Among all nitrogen sources, beef extract gave the highest yield of enzyme (158 U/ml) so that in the next experimental series, beef extract was added at various concentrations (0.25- 1.25 %) and GGH production was determined (Fig 2f). The concentration of 1 % gave the maximum enzyme production (190 U/ml). Similar results were observed by Simair *et al.* [38]. Higher or lower concentrations were equally harmful, causing enzyme repression. An increase in nitrogen concentration caused production of protease which ultimately inhibits the GGH production [35].

3. 4. Influence of various surfactants on the GGH production

Influence of various surfactants such as EDTA, SDS, Tween 80 and Triton X-100 on the GGH production is presented in Figure 3a. Tween 80 was found to be the best for the maximal GGH production (220 U/ml) and effects of different concentrations (0.01-0.07 %) of this surfactant are presented in Figure 3b. Maximal enzyme production was observed at 0.05 % Tween 80 (239.1 U/ml). This result might be due to the increase in permeability of the cell membrane [39].

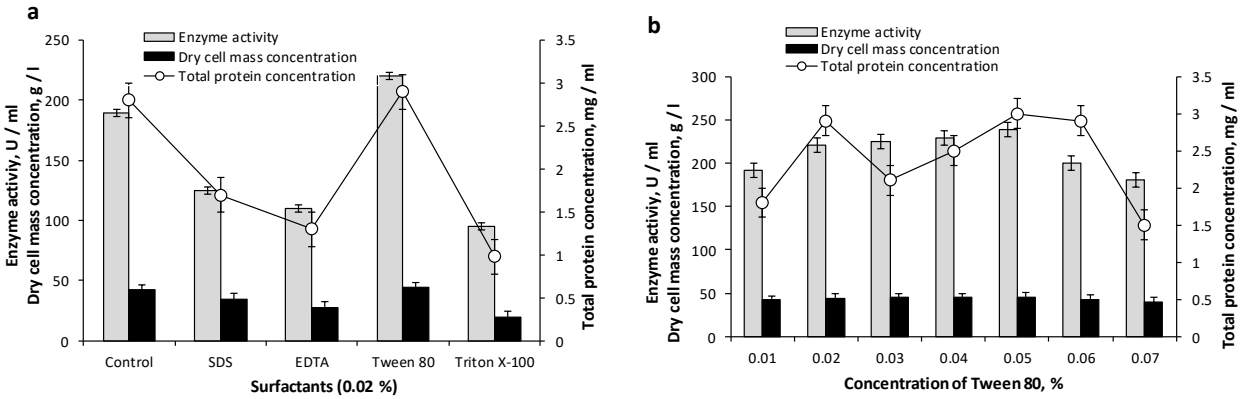

Figure3: Influence of surfactants and metal ions on the production of 1, 4-á-D-glucan glucanohydrolase by mutant co-culture of Bacillus subtilis EMS-10 and Bacillus amyloliquefaciens EMS-10; (a) Different surfactants, (b) concentrations of Tween 80.

4. CONCLUSIONS

Results obtained in the present study indicate that mutagenesis, optimization and utilization of synergistic phenomena of a mutant bacterial co-culture enhance the GGH production as compared to the wild co-culture. The selected mutant co-culture of *Bacillus subtilis* EMS-10 and *Bacillus amyloliquefaciens* EMS-10 may be a potential candidate for industrial utilization.

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SAŽETAK

Spontana mutageneza (pojava mutacija) i optimizacija procesa ko-kulture bakterija za hiperprodukciju 1,4-α-D- glukan glukanohidrolaze (α-amilaze) primenom submerzne fermentacije

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Eksponencijalni porast primene 1,4-α-D-glukan glukanohidrolaze (GGH) u različitim oblastima, doveo je do zahteva za poboljšanje njene proizvodnje, kako u kvalitativnom, tako i u kvantitativnom smislu, kroz poboljšanje bakterijskih sojeva koji se koriste za proizvodnju. U ovom radu, *Bacillus subtilis* LCBT-15 i *Bacillus amyloliquefaciens* LCBT-20 su podvrgnuti fizičkoj, kao i hemijskoj mutagenezi u cilju poboljšanja proizvodnog potencijala GGH. Primena UV svetlosti i etidijum bromida nisu uzrokovale značajno povećanje proizvodnje enzima. Međutim, ko-kultura 10 tretirana etil metan sulfonatom (EMS) dovela je do povećanja proizvodnje GGH 1,3 puta, za razliku od divlje ko-kulture. Ispitani su različiti fizičko-hemijski parametri, uključujući vrstu medijuma za fermentaciju, brzinu fermentacije, temperaturu, pH, azot, izvore ugljenika i površinske aktivne materije. Sastav M7 medijuma optimizovan je za proizvodnju GGH posle 48 h inkubacije na 37 °C pri pH 6. Optimalna veličina inokuluma iznosila je 3,5 ml (1×10^6 ćelija / ml) u 50 ml medijuma. Najbolji izvori ugljenika i azota su laktoza (2,5 %); amonijum hlorid (1,75 %) i goveđi ekstrakt (1 %), respektivno. Optimalna proizvodnja GGH (287 U/ml) dobijena je kada je medijum dopunjen sa 0,05 % Tween 80. Ovim istraživanjima dodatno je razjašnjen sinergistički efekat mutirane bakterijske ko-kulture na poboljšanje proizvodnje GGH.

Ključne reči: mutacija, etil metan sulfonat, ko-kultura, 1, 4-α-D-glukan-glukanohidrolaza, *Bacillus*