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Dextranase: Hyper production of dextran degrading enzyme from newly isolated strain of *Bacillus licheniformis*

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ABSTRACT

Dextranase, 6-alpha-D-glucan 6-glucanohydrolase catalyzes the degradation of dextran (polymer of D-glucose) in to low molecular weight fractions. Dextranolytic bacterial strains were isolated from various natural sources and plate assay methods were developed for screening of highest extracellular dextranase producing isolate. *Bacillus licheniformis*, identified on the basis of taxonomic characterization was subjected to UV radiation and highest enzyme producing mutant obtained led to 7 times more dextranase production than wild. Optimization of major physico-chemical parameters affecting enzyme production; including medium composition, pH, cultivation time and temperature revealed that maximum enzyme production was obtained in a self designed medium (pH 6.0) containing 1% Dextran 5000 Da, after 24 h culture incubation at 37 °C. Dextranase reported in this study is of great commercial importance as it is strictly inducible in nature and *B. licheniformis* being non-pathogenic removes the safety concerns associated with production of dextran fractions for clinical and pharmaceutical usage.

1. Introduction

Dextran is a long chain carbohydrate polymer (α-1,6-glucosidic linkages), synthesized from glucose by an enzyme dextransucrase (EC 2.4.1.5). Various bacteria, fungi and other species are capable to produce dextran as an exopolysaccharide (Khalikova, Susi, & Korpela, 2005). Dextran is hydrolyzed to low molecular weight fractions, which are used in many biotechnological processes. Conventionally, high molecular weight dextran is hydrolyzed by acid treatment to generate low molecular weight fractions (Renfrew & Cretcher, 1949). Through industrial revolution acid hydrolysis has been replaced by a more cost effective and environmental friendly enzyme technology. Dextranases, 6-alpha-D-glucan 6-glucanohydrolase (EC 3.2.1.11) are the enzymes used for hydrolysis of high molecular weight dextran to low molecular weight fractions (Lee & Fox, 1985; Sidebotham, 1974).

Different fractions of dextran are of significant commercial interest in drug formulations, vaccines, cryoprotectants and stabilizers in the food industries, cosmetic products and as separating gels in research studies (Khalikova et al., 2005). Specific molecular weight fractions of dextran generated by dextranase are used to restore blood volume in patients suffering shock as a result of severe blood loss (Ingelman, 1948; Kim & Day, 1994; Mehvar, 2000). In addition to using dextranases for processing dextran, the enzyme itself is of great significance in beverage industry for the treatment of juices, syrups and in oral care products such as mouth washes and dental paste for removal of dental plaque (Jiménez, 2005; Khalikova et al., 2005). Dextran degrading organisms have been reportedly isolated from natural environment as well as from harsh environment (Hoster, Daniel, & Gottschalk, 2001; Mahmoud & Helmy, 2009). Cloning and expression of dextranase encoding gene have also been reported (Lee et al., 2010; Pons, Garcia, & Castellanos, 2000). Current study was focused on exploitation of indigenous microbial flora for isolation of dextranolytic bacterial specie and to enhance the production of extracellular dextranase using UV mutation of wild strain and optimization of physico-chemical parameters for the enhanced production of enzyme.

2. Materials and methods

2.1. Isolation and screening of dextranase producing strain

Microorganisms capable of hydrolyzing dextran were isolated from various natural habitats such as molasses, garden soil and deteriorating sugar cane chunks from sugar cane field. Samples were serially diluted in sterile distilled water and 100 μl from 10^{-6} dilution was plated on media reported by Khalikova, Susi, Usanov, and Korpela (2003) for isolation of bacterial specie using spread plate technique. After incubation at 37 °C for 24 h, plates were flooded with Gram's Iodine solution to visualize hydrolytic

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zone around colonies capable of hydrolyzing dextran. Colonies showing hydrolytic zones were purified by repeated streaking on same medium. Highest enzyme producing strain was identified on the basis of morphological and biochemical analysis, according to "Bergey's Manual of Determinative Bacteriology" (Holt, Krieg, Sneath, Staley, & William, 1994). Pure culture was preserved on nutrient agar slants and stored at 4 °C for further studies.

2.2. Dextranase assay

Dextranase activity was determined by measuring reducing sugar liberated during enzyme–substrate reaction (Nelson, 1944). Cell free filtrate (0.5 ml) was incubated with 1.0 ml substrate (0.5% Dextran, MW5000 Da) solubilized in 0.1 M citrate phosphate buffer (pH 4.5) at 35 °C for 15 min.

One unit of dextranase was defined as the amount of enzyme required to catalyze the formation of 1 μmol of maltotriose at standard assay condition.

2.3. Strain improvement for higher dextranase production using UV mutation

Freshly grown cells of *Bacillus licheniformis* were diluted in normal saline (0.85% NaCl) to reach an O.D. of 0.4 at 600 nm. Suspension was then serially diluted and 0.1 ml was plated on nutrient agar plates using spread plate technique. Plates were UV-irradiated from a distance of 20 cm for various time intervals and kept in dark for 02 h at room temperature and then incubated at 37 °C for 72 h. Colonies appeared were revived initially in 0.5% dextran media and then in 1% dextran media. Enzyme activity of all mutants was estimated using standard assay procedure and the strain producing higher enzyme yield was selected for further studies.

2.4. Selection of medium for dextranase production

Several reported media were used for higher dextranase production from *B. licheniformis* (Fukumoto, Tsuji, & Tsuro, 1971; Khalikova et al., 2003; Simonson, Lamberts, & Shklair, 1972; Subasioglu & Cansunar, 2010). After inoculation, 100 ml of each medium was subjected to incubation for 24 h at 37 °C and after fermentation, broth was centrifuged at 12,000 rpm for 15 min at 4 °C and enzyme activity was performed by standard assay procedure. A medium for further studies was designed for maximum dextranase production having the composition (g/dl) Dextran (MW 5000 Da) 10.0, yeast extract 1.0, peptone 10.0, K_2HPO_4 1.0, $MgSO_4$: $7H_2O$ 0.2 and NaCl 3.0 keeping pH of medium at 6.0.

2.5. Optimization of carbon source for dextranase production

Various carbon sources such as glucose, sucrose, starch and dextran of different molecular weights (dextran 5, dextran 10, clinical grade, high molecular weight) were added in growth medium.

2.6. Optimization of nitrogen source and concentration for dextranase production

Various organic and inorganic nitrogen sources such as yeast extract, tryptone, peptone, urea, potassium nitrate, sodium nitrate and ammonium sulfate were added in growth medium before sterilization for maximum production of dextranase.

2.7. Optimization of incubation temperature for dextranase production

The temperature optimum for maximum yield of active enzyme was determined by incubating bacterial culture flasks at various temperatures ranging from 20 °C to 60 °C for 24 h.

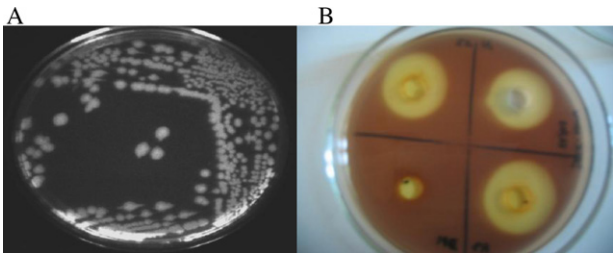

Fig. 1. Growth of *Bacillus licheniformis* on nutrient agar plate (A), plate assay showing clear zone of dextran hydrolysis around well (B).

2.8. Optimization of medium pH for dextranase production

Medium pH was varied within a wide range using 1 N HCl and 1 N NaOH prior sterilization. To estimate optimum pH for enzyme production, enzyme activity of cell free broth of every pH medium was calculated by standard assay procedure.

2.9. Optimization of incubation time for dextranase production

Suitable incubation time required by bacterial strain for maximum enzyme production was investigated by subjecting inoculated culture flask at different time intervals (24–96 h). Optical density of nutrient medium was also measured at 600 nm to estimate its biomass content.

3. Results and discussion

3.1. Isolation of dextranase producing bacterial strain

Dextranase producing organisms were isolated from different sources and grown on medium containing dextran as sole carbon source with other medium components and after 24 h of incubation, dextranolytic bacteria were selected on the basis of clear zone of dextran hydrolysis (Fig. 1). Based on this screening procedure, only the bacterial strains capable of producing extracellular dextranase were selected for pure culture study by repeated culturing on same medium. According to morphological and biochemical analysis, highest dextranase producing strain was characterized as *B. licheniformis*.

3.2. Strain improvement for higher enzyme production

UV mutagenesis of *B. licheniformis* led to development of 35 mutants among which 07 mutants demonstrated higher enzyme activity as compared to wild (15 U/ml/min). After UV irradiation, survival of cells in percent with respect to exposure time duration is shown in Table 1. From screening and repeated culturing of mutant strains, one strain was found to be consistently producing higher enzyme yields (105 U/ml) than wild. It was reported that for enhanced dextranase production, *Lipomyces starkeyi* (Kim & Day, 1995), *Penicillium notatum* (Szczodrak, Pleszczynska, & Fiedurek, 1994) and *Penicillium funicolosum* (Abdel-Aziz, Talkhan, & Janson,

Table 1
Percent survival of *Bacillus licheniformis* cells after UV exposure for different time intervals.

UV exposure time (s)	Number of colonies	Survival (%)
Control	118	Nil
5.0	8	6.70
15.0	6	5.00
30.0	1	0.84
60.0	1	0.84
120.0	0	Nil

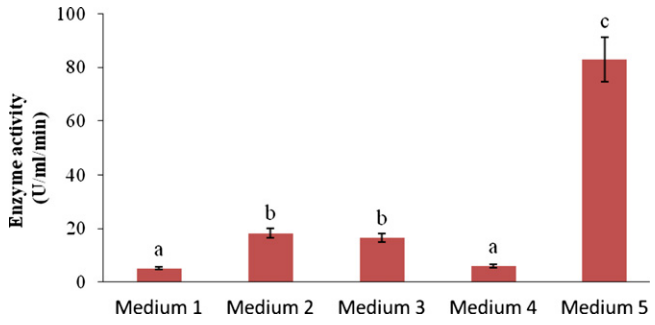

Fig. 2. Effect of different medium on dextranase production from *Bacillus licheniformis*. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

2007) had also been subjected to mutagenesis and 189% increased in dextranase production was found as compared to wild strain of *P. funicolosum* (Abdel-Aziz et al., 2007).

3.3. Media selection for optimal enzyme production

Various reported media were used for optimal bacterial growth and higher enzyme yield. Maximum enzyme production and bacterial growth was found in newly designed medium as compared to reported media (Fig. 2).

3.4. Optimization of carbon source and concentration

Different carbohydrate sources such as glucose, sucrose, starch and dextran (with varying molecular weights) were incorporated in fermentation medium as only carbon source. It was found that higher dextranase production was achieved when medium is supplemented with dextran as only carbon source and no enzyme production was found in medium having glucose, sucrose or starch. This result clearly suggested that enzyme production is inducible and also highly substrate specific. It was found that maximum dextranase production was achieved when low molecular weight dextran (MW 5000) was incorporated in the medium and dextranase production is inversely proportional to polymer length and extent of branching (Fig. 3). It was already reported that bacterial growth increases by using dextran as carbon source which ultimately increases dextranase production (Fukumoto et al., 1971; Mahmoud & Helmy, 2009). Various other microorganisms such as *Bacteriodes oralis*, *Bacillus circulans*, *Streptococcus sobrinus*, *Streptomyces anulatus* and many yeast and fungal specie hydrolyzed different molecular grades and forms of dextran ranging from 40 Da

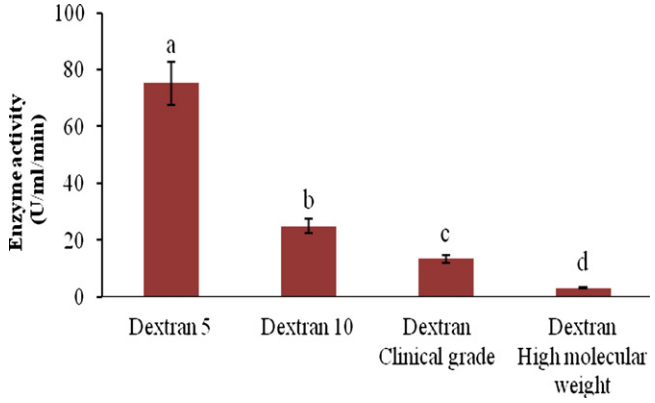

Fig. 3. Effect of dextran (different molecular weight) on dextranase production from *Bacillus licheniformis*. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

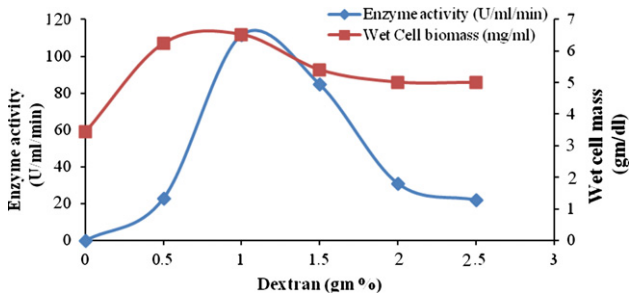

Fig. 4. Effect of various dextran concentrations for maximum dextranase production.

to 200,000 Da and insoluble matrices like sephadex G-25 to G-200 (Igarashi, Yamamoto, & Goto, 2001; Khalikova et al., 2003; Kolenbrander et al., 2002; Kubic, Sikora, & Bielecki, 2004; Mahmoud & Helmy, 2009).

Maximum enzyme production was achieved when 1.0 g % dextran-5 (5000 Da) was used as a carbon source in fermentation medium. It was also found that increased in 0.5% concentration of dextran-5 from 1.0% to 1.5%, enzyme production decreases up to 26% and if the concentration decreases from 1.0% to 2.0%, enzyme production decreases up to 72%. The reason might be due to excess accumulation of dextran in medium which increases the viscosity and ultimately hinders the bacterial growth, resulting in decreased production of dextranase (Fig. 4).

3.5. Optimization of nitrogen source and concentration

Different organic and inorganic nitrogenous sources play key role in the dextranase production. It was found that yeast extract, tryptone and peptone supported higher enzyme production as compared to other nitrogen sources such as urea, ammonium nitrate, ammonium phosphate and potassium nitrate at different concentrations (Fig. 5). Moreover when yeast extract and peptone were added in media simultaneously, dextranase production increased to 61.3% which suggested that single nitrogen source is insufficient for higher enzyme yield as also reported by Qader, Bano, Aman, Syed, and Azhar (2006) in case of amylase production from *Bacillus subtilis* KIBGE-HAS.

Once the suitable source of carbon and nitrogen was selected, concentrations of other medium components were also optimized for higher dextranase production. Variation in concentration of $MgSO_4$, NaCl and K_2HPO_4 suggested 0.02%, 0.3% and 0.1% respectively being optimum for maximum production of enzyme.

3.6. Optimization of incubation temperature and pH of medium

By subjecting *B. licheniformis* to different incubation temperatures ranging from 20 to 60 °C, it was found that maximum

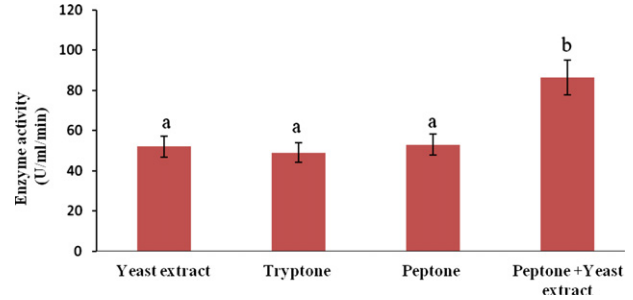

Fig. 5. Effect of nitrogen sources on dextranase production from *Bacillus licheniformis*. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

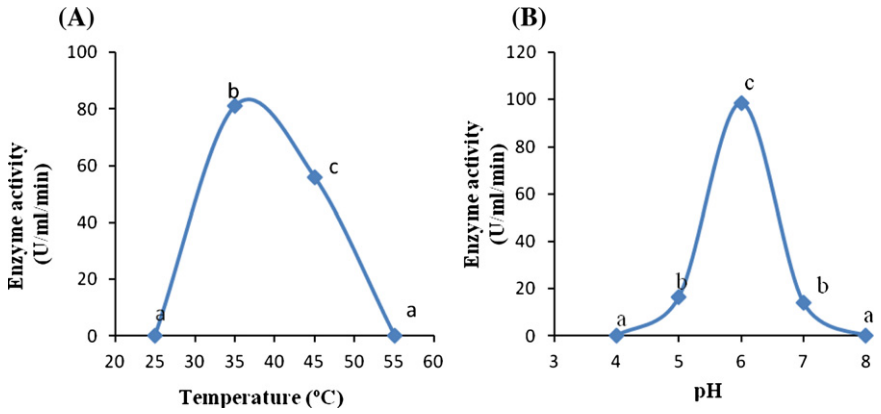

Fig. 6. Effect of fermentation temperature (A) and medium pH (B) on dextranase production from *Bacillus licheniformis*. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

dextranase production was achieved at 35 °C (Fig. 6A). Varying the temperatures from this range adversely affected the enzyme production and loss of 31% enzyme production was observed at 45 °C and further more increased in temperature up to 60 °C resulted in complete loss of enzyme production. This loss of enzyme production is might be due to low multiplication rate of the bacteria which ultimately decreased the enzyme production. Subasioglu and Cansunar (2010) reported decrease in enzyme production due to the decrease in fungal biomass when incubated at suboptimal temperature.

Maximum dextranase production was achieved when optimal pH of medium was kept at pH 6.0 before sterilization. As the pH of the medium increased or decreased from optimal pH, a sharp declined in enzyme production was observed (Fig. 6B). These results are in agreement with previously reported results of dextranase produced by fungal specie, when medium pH was kept at range from 5.5 to 6.0 (Das & Dutta, 1996; Shimizu, Unno, Ohba, & Okada, 1998).

3.7. Optimization of incubation time

The relationship between time required for growth of organism and maximum enzyme yield is shown in Fig. 7. It was found that maximum dextranase production (89 U/ml/min) was achieved after 24 h of incubation and gradually decreased onwards. This pattern of activity can be explained by inference of Arnold, Nguyen, and Mann (1998) that oligosaccharides generated by dextranase enzyme are taken up by cells due to exhaustion of nutrients in the medium.

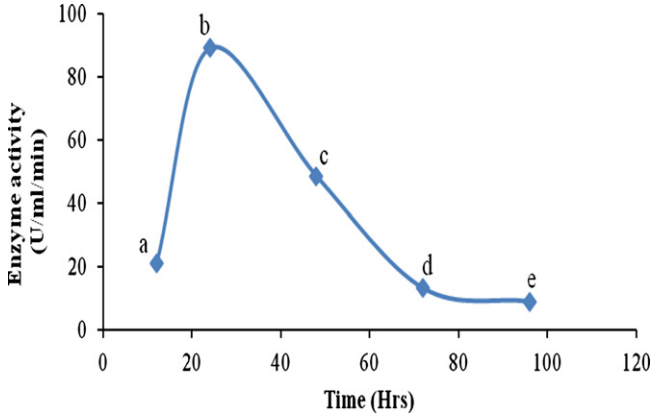

Fig. 7. Effect of fermentation time on dextranase production from *Bacillus licheniformis*. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

4. Conclusion

This study deals with enhanced production of a dextranase from newly isolated *B. licheniformis*. Strain was UV mutated for enhanced dextranase production and selected mutant was then subjected to varying experimental procedures. Physico-chemical parameters which employed drastic effect on enzyme production such as suitable nutrient medium, substrate, nitrogen sources, growth temperature of organism, pH of growth medium and incubation time required for dextranase production were optimized. The dextranase reported in this study is an extracellular, inducible and highly substrate specific which degraded low molecular weight dextran. Analysis of reaction's end products and kinetic parameters may reveal exact action mechanism of the enzyme. The reported bacterium could be an ideal candidate for industrial production of dextranase as it is a non-pathogenic strain which grows at mild conditions and produces extracellular, highly substrate specific dextranase which cannot only be used in its purified form but also in crude and partially purified forms as well for the removal of dextran contamination.

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