Journal of Advances in Microbiology

3(2): 1-6, 2017; Article no.JAMB.30911
ISSN: 2456-7116

Chemical Mutagenesis of *Bacillus subtilis* for Improved Mannanase Biosynthesis

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Authors' contributions

This work was carried out in collaboration between all authors. Author BSA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SOO managed the analyses of the study. Authors YDO and OOO managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2017/30911
Editor(s):
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Complete Peer review History: http://www.sciencedomain.org/review-history/19147

Original Research Article

Received 7th December 2016
Accepted 27th April 2017
Published 29th May 2017

ABSTRACT

The aim of the present study was to isolate bacterial associated with abattoir waste water, compost saw dust, soil and water samples from Ilaje Lake, Ondo State, Nigeria. The microbial isolates were identified using standard microbiological method. The bacterial isolates were screened for mannanase production. Mannanase activity was determined by dinitrosalicylic acid (DNSA) method while protein concentration in the fermentation broth was quantified by Lowry's method. Isolate designated 2k tentatively identified as *Bacillus subtilis* had the highest mannanase activity. The isolate with highest mannolytic activity was then subjected to different mutagens. The mutants of *Bacillus subtilis* generated were screened for mannanase production in comparison with the wild type in submerged state fermentation. All the mutant strains generated from *B. subtilis* had their mannolytic activities repressed in comparison with the wild strain. Out of mutants screened, mutant designated CH017 have the highest mannolytic activity 1.20 U/mg. The mannanase activity produced by CH017 was approximately 44% lower than the wild strain. The

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pretreatment of *B. subtilis* with nitrous acid caused enzyme repression. Therefore, another chemical mutagen should be worked upon whether it would result in appreciable yield of mannanase.

Keywords: Chemical mutagens; mannanase; Bacillus subtilis; environmental wastes; submerged fermentation.

1. INTRODUCTION

Microbes are known to be major source of industrial enzymes. The demand for industrial enzymes is on increase driven by a growing need for sustainable solutions [1]. Mannanase is an enzyme that breaks down compounds known as mannanes. Mannanes are the major constituents of the hemicellulose fraction in softwoods and show widespread distribution in plant tissues. Mannanases has been foremost used as additives in feed containing soya and maize intended for broiler hens and swine with more relevant in the improvement in their nutrient uptake and weight gain [2]. The addition of β-mannanases into animal feed generates manno-oligosaccharides, mannotriose and mannobiose as well as small amounts of mannose via the hydrolysis of mannan [3]. Microbial mannanases are mainly extracellular and can act in wide range of pH and temperature which has been found useful in pulp and paper, pharmaceutical, food, feed and textile industries [4]. Microbial enzymes are known to play a crucial role as metabolic catalysts, leading to their use in various industries and applications. The preference of microbial enzymes to other sources of enzymes might be as a result of their easy genetic manipulation, grown in large culture medium and replication [5]. Microbial enzymes have been applied in various fields, including technical use, food manufacturing, animal nutrition, cosmetics, medication, and as tools for research and development [6]. With the improved understanding of the enzyme production, biochemistry, fermentation processes, and recovery methods, an increasing number of industrial enzymes can be more applicable on industrial scale [7].

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples for microbial analysis were collected from abattoir, saw mill and Ilaje Lake, Ondo state, Nigeria. The samples were transported to the Microbiology Laboratory, Federal University of Technology, Akure, Nigeria and were used as source for the isolation of mannanase-producing bacteria [8].

2.2 Isolation of Mannanase-producing Bacteria

Pour plate method was used for the isolation of mannanase-producing bacterial from different sources. One gram of solid sample and 1mL water sample was suspended in 9 mL sterilized distilled water. One percent (v/v) of the solution was transferred into 20 mL sterilized isolation medium containing Locust Bean Gum (LGB) (1%) [9]. The bacterial cells were grown under aerobic condition by shaking at 150 rpm for 24 hours at 37ºC. The culture broths were serial diluted and pour plated in isolation medium containing LBG using appropriate dilution factors. The cells were allowed to grow at 37ºC for 18-24 hours and the total bacterial counts from the samples were counted [10].

2.3 Primary Screening of Mannanase-producing Bacteria

The culture broth from the enumeration step was serially diluted and spread on isolation medium containing 1% (w/v) Locust Bean Gum (LBG) and cells were allowed to grow at 37±2ºC for 18-24 hours. The colonies with clear zones of mannanase activity were observed [9,10]. The positive isolates were selected and kept in 20% glycerol for further study.

2.4 Chemical Mutagenesis

The modified method of [11] was employed to mutagenize the bacterial cell. Nitrous acid (0.1Molar sodium nitrite in phosphate buffer, pH 5.0) was used as mutagen. Broth culture of the organism was incubated for 18 to 24 hours at 35°C. The culture was centrifuged at 6000 rpm for 15 minutes and the sediment obtained was washed twice with normal saline. Then, 0.8 mL nitrous acid was added to 2.0 mL the washed cells. The mixture was incubated for 1 hour at 35°C, washed twice with normal saline at dilution ratio 1:10 in a minimal medium containing 1% LBG, 0.1% peptone, 0.1% yeast extract, 0.2%

$NaNO_3$, 0.05% KCl, 1.4% KH_2PO_4, 0.06% $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ traces, pH 6.8. The culture was incubated at 35°C for 24 hours to allow segregation of the mutants. Then, 0.2 mL of growing cells was plated out on nutrient agar plate using sterile glass spreader and incubated at 35°C for 24 hours. Twenty isolated mutant colonies were identified properly and transferred to a fresh nutrient agar plate by replica plating technique. These mutants and the wild type strain of *B. subtilis* were used for mannanase biosynthesis.

2.5 Secondary Screening of Mannanase-producing Bacteria

The mutants and the wild type of *B. subtilis* were screened for their ability to produce mannanase under submerged state fermentation. Enzyme production was performed in 250 mL flask containing 50 mL of enzyme producing medium modified by [10]. The medium composition was as followed: 1% LBG, 0.1% peptone, 0.1% yeast extract, 1.4% KH_2PO_4, 0.06 % $MgSO_4.7H_2O$, and 1% inoculums, pH 6.8. The flasks were incubated at 35ºC for 24 hours on a rotary shaker (Gallenkamp) at 120 rpm. Then, the culture broth was centrifuged at 6000 rpm using cold centrifugation at 4ºC for 15 minutes. The supernatant was collected and kept at -20ºC for further study.

2.6 Assay for Mannanase Activity

Mannanase activity of supernatant collected at the end of incubation period was determined using Spectrophotometer (Lab-Tech Digital Colorimeter) by the method of [10]. The reaction mixture contained 1mL LBG (1%) dissolved in 50Molar phosphate buffer pH 7.0 and 1mL of enzyme solution. The control tube contained the same amount of substrate and 1mL of enzyme solution heated at 100ºC for 15 minutes. Both the experimental and control tubes were incubated 40ºC for 5 minutes. At the end of the incubation period, tubes were removed from the water bath (Lamfield Medical England Model DK-600), and the reaction was stopped by the addition of 2 mL of 3, 5-dinitrosalicylic acid (DNSA) reagent per tube [10]. The tubes were incubated for 10 minutes in a water bath for colour development and were cooled rapidly. The mannanase activity of reaction mixture was measured against a reagent blank at 540 nm. The released mannose due to mannanase activity was determined by DNS method [12]. One unit of mannanase activity is defined as the amount of enzyme producing one micromole of mannose under the assay conditions.

2.7 Protein Estimation

Protein in the medium was determined using Bovine Serum Albumin (BSA) as standard [13].

3. RESULTS AND DISCUSSION

3.1 Populations of Bacteria from Different Sources

Table 1 showed total bacterial counts from different sources. Ilaje lake water (5.11×10^9 cfu/ml) showed the highest number of bacterial population, while saw dust (4.18×10^6 cfu/g) recorded least bacterial counts. The increase in the bacterial population might be due to the nutrient availability in the water body, intrusion of contaminant from the surrounding environment and favourable environmental conditions while the decrease in the bacterial population from saw dust might be due to quality control check observed at the point of collection and handling process.

Table 1. Total bacterial count from different source

Sources	Bacterial count
ILS	2.2×10^9(cfu/g)
ABT	9.05×10^8(cfu/mL)
ILW	5.11×10^9(cfu/mL)
SD	4.18×10^6(cfu/g)

Keys: ILS = Soil sample from Ilaje lake, ILW = Water sample from Ilaje lake
ABT = Water sample from the abattoir, SD = Sawdust sample

3.2 Screening of Mannanase Producing-bacteria in Submerged State Fermentation

The quantitative determination of mannanase degrading enzyme is shown in Table 2. The bacterial isolates obtained from different sources exhibited different mannanase activities in media supplemented with LBG. The highest specific mannanase activity 2.75 U/mg was produced by bacterial isolate 2K sourced from Ilaje Lake, while the lowest value of 0.41 U/mg was obtained by isolates 2E1 and 2L1. Protein content rangedfrom 1.75l - 6.44 mg/mL with the highest protein content produced by 2K2 obtained from Ilaje Lake. Therefore, bacterial isolate 2K was selected for mutagenic studies

(using nitrous acid as mutagenic agent) because of its highest mannanase-producing potential. The mannanase production in LBG medium had been reported for *Bacillus circulans* [10], *Chryseobacterium indologenes* [14], *Bacillus* sp. MG-33, *Bacillus amylolequifaciens*10A1, *Bacillus* sp., *Aspergillus niger* [15,16], *Sclerotium rolfsii* [15], *Trichoderma* sp. [16] and *Scopulariopsis candida* [17, 18], *Trichosporonoides oedocephalis* [19]. The variation between these isolates for the production of mannanase on LBG media could be attributed to the ability of their source of isolation and slight genetic make up to secrete active mannanase with high diffusion rate [14, 19] The variation in protein content generated by each of the strains in submerged state fermentation could be due to the production of variety of enzymes (amylases, cellulases, protease and xylanases) in addition to the enzyme been examined in this study. Presumably, the protein from bacterial cells and metabolites rich in protein might interfere with mannanase production causing variation in protein contents, since the protein assay could only identify accumulated protein in enzyme production medium [19,20].

Strain improvement is an essential part of process development for bio-products formation. Developed strains can reduce the costs with increased productivity and can possess some improved and desirable properties. Such improved strains can be achieved by mutagenesis in the natural strain and subsequent screening. Thus a major effort of industrial research in producing enzymes is directed towards the screening programs. Mutation is the primary source of all genetic variation and has been used extensively in industrial improvement of enzyme production [19]. The repression of mannanase biosynthesis by mutants generated from this study contradicted the findings of [19] that reported an appreciable mannanase production by a mutant developed from *Klebsiella edwardsii* using similar mutagenic techniques. Many researchers had reported strains improvement using different chemical mutagens for industrial relevant enzymes production. [21] reported strain's improvement with the use of ethyl methyl suphonate, n-methyl-N'-nitro-N-nitroso guanidine and ethidium bromide [6]. However, scanty information is available for the improvement of strain for mannanase production. The variation in mannanase production between mutant strains could be due to some factors like damaged DNA and differences in their ability to repair damaged genes [19].

Table 2. Production of extracellular mannanase by bacterial isolates from different sources

Sources	Isolate code	Mannanase activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)
ILS	1A1	4.43	5.08	0.87
	1C1	2.14	5.12	0.42
	1C1 2	3.23	5.61	0.58
	1C4	3.13	2.61	1.2
ILW	2E1	2.48	6.03	0.41
	2F2	2.89	6.30	0.46
	2G1	10.16	4.17	2.44
	2H3	3.43	2.73	1.36
	2J1	2.06	1.78	1.16
	2K	13.07	4.75	2.75
	2K11	3.62	4.03	0.9
	2L1	1.98	4.88	0.41
ABT	3B	2.60	3.03	0.86
	3C2	2.46	3.89	0.63
	3D1	3.37	2.06	1.64
	3E	3.63	3.67	0.99
	3E2	4.00	1.75	2.29
SD	4B	2.92	5.97	0.49
	4D5	2.87	4.35	0.66
	4E	6.13	5.03	1.22
	4F	2.19	4.73	0.46

Keys: ILS = Soil sample from Ilaje lake, ILW = Water sample from Ilaje lake, ABT = Water sample from the abattoir, SD = Sawdust sample

Table 3. Mannolytic activity of wild and mutant strains of *Bacillus subtilis*

Mutants	Mannanase activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)
CHO1	1.18	21.80	0.05
CHO2	9.42	16.40	0.57
CHO3	16.35	20.89	0.78
CHO4	13.45	18.01	0.75
CHO5	16.72	18.54	0.90
CHO6	13.19	15.68	0.84
CHO7	15.64	14.83	1.06
CHO8	15.15	16.71	0.91
CHO9	16.90	17.53	0.96
CHO10	15.58	19.14	0.81
CHO11	14.18	17.18	0.83
CHO12	15.48	19.48	0.80
CHO13	15.50	18.78	0.83
CHO14	15.02	19.08	0.79
CHO15	17.80	18.22	0.98
CHO16	17.27	17.00	1.02
CHO17	17.73	14.83	1.20
CHO18	15.03	15.36	0.98
CHO19	15.73	21.22	0.74
CHO20	16.60	19.13	0.87
Wild strain	13.07	4.75	2.75

4. CONCLUSION

There have been efforts to generate improved microorganisms with high ability to produce industrial enzymes that can meet up with industrial demand of these enzymes. The bacteria encountered from different sources elaborated an appreciable mannanase production with 2K showing the highest mannanase activity 13.07 U/mL and 2L1 showing the mannanase activity 1.98 U/mL and this could be exploited in many industrial processes where they are been utilized. However, no potential mannanase producers have been developed through either chemical or physical mutagenesis till date.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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