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ORIGINAL ARTICLE

A thermostable and acidophilic mannanase from *Bacillus mojavensis*: its sustainable production using spent coffee grounds, characterization, and application in grape juice processing

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Abstract
The utilization of enzymes in various industrial applications has attracted increasing attention in recent years. However, their production costs and instability in extreme environments are two significant factors that limit their use in industry. Mannanase production from *Bacillus mojavensis* using spent coffee grounds (SCGs), which are inexpensive and most abundant domestic industrial waste, was increased about 32-fold with optimization of submerged fermentation (SmF) medium using Plackett–Burman design (PBD) and response surface methodology–Box–Behnken design (RSM–BBD). Moreover, BmMan production in a 5-L stirred bioreactor resulted in an enzyme titer of 32.55 U/mL. BmMan was partially purified 2.28-fold with 63.01% yield using ultrafiltration and ammonium sulfate precipitation techniques. The molecular weight of partially purified mannanase (BmMan) was estimated by zymography to be 24 kDa. Biochemical characterization of BmMan showed that optimal pH and temperature for catalysis were 4.0 and 70 °C, respectively. BmMan showed a half-life of 40.8, 150.2, 210.2, and 387.6 min at 80, 70, 60, and 50 °C, respectively. Subjecting to bivalent cations and chemicals, Ag^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, DTT, Triton X-100, SDS, and β-mercaptoethanol enhanced BmMan activity. Additionally, BmMan was compatible with methanol, DMSO, isopropanol, and butanol. The clarity and nutritional value of grape juice were improved after the treatment with the enzyme. Ultrafiltrate obtained by passing cell-free culture liquid through a 10-kDa molecular weight cutoff (MWCO) membrane exhibited antimicrobial and antioxidant activity without showing genotoxicity. To the best of our knowledge, this is the first report describing the thermostable and acidophilic mannanases produced by *Bacillus* taxa and the first study suggesting the multiple uses of SCGs.

Keywords Mannanase · Thermostable · Acidophilic · *Bacillus mojavensis* · Spent coffee grounds · Juice processing

1 Introduction

The intensive use of chemicals in industrial applications and daily life causes global threats such as climate change, environmental damage, a series of health problems, and biodiversity loss. Therefore, societies are faced with the urgent need to change their production and consumption habits. One of the solutions to mitigate current threats is using enzymes, which are eco-friendly, safe, and highly specific, as an alternative to chemical catalysts. Endo-1,4-β-mannosidases, commonly known as mannanases (EC 3.2.1.78), act on mannans by randomly cleaving the backbone to liberate short β-1,4-mannooligomers and produce new chain ends [1]. Mannanases are classified into glycoside hydrolase (GH) families 5, 26, 113, and 134, and most of them exhibit (β/α)8-barrel fold structure. They can be used in bioenergy, food, paper, and feed industries for various purposes such as oil drilling, bio-bleaching of pulps, clarification of fruit juices, saccharification of lignocellulosic materials, removal of impurities in fabrics, preparation of instant coffee, and improving feed quality [2]. Mannanases are also used in functional foods through the enrichment of mannooligosaccharides, which can function as antitumor agents and immune modulators [3, 4].

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Mannanases have been successfully produced by various bacterial strains. However, many of them have limited use in food and feed industries since their activities are significantly reduced in high-temperature and acidic environments. Till now, a few bacteria including *Clostridium cellulovorans* [5], *Paenibacillus* sp. CH-3 [6], *Streptomyces* sp. NRRL B-24484 [7], *Thermoanaerobacterium aotearoense* SCUT27 [8], and *Microbacterium* sp. CIAB417 [9] have been described as thermostable and/or acid-tolerant mannanase producers. Therefore, investigating new or novel bacterial sources of mannanase producers is of industrial interest. *B. mojavensis* strains are aerobic, Gram-positive, and endospore-forming bacteria that can grow at a temperature range from 5 to 55 °C under acidic conditions [10]. They resist various environmental stresses due to their endophytic nature, and thus, they can survive in different habitats. Therefore, they show excellent biosynthetic, genetic, and functional versatility. These characteristics make them promising candidates for producing enzymes stable in harsh conditions.

One of the most important factors limiting the production of enzymes on an industrial scale is the cost of expensive inducers and un-optimized medium in the process. Industrial and agricultural wastes can be used as inducers to reduce the processing cost. Spend coffee grounds (SCGs) are waste from the brewing process of coffee, the world's most traded commodity [11]. Although SCGs have high nutritional value, they cannot be used as feed and fertilizer due to their toxicity against animals and plants; as a result, they are widely dumped in landfills [12, 13]. The main chemical component of SCGs is hemicellulose containing xylans and mannans [14]. Therefore, they are a no-cost and plentiful resource for mannanase production.

So far, a huge amount of studies have been focused on microbial enzyme production using waste-based feedstock. These have not been included in a life cycle assessment (LCA) perspective, though. Various bioactive compounds such as phenols, organic acids, and oligosaccharides can be release from cells or/and SCG to culture liquid during bioprocessing. These soluble compounds can be recovered by ultrafiltration prior to purification of the enzyme and used as antimicrobial and antioxidant agents.

In the present study, the production of thermostable and acidophilic mannanase using *B. mojavensis* TH309 was achieved. The production using SCGs was optimized by response surface methodology approaches. The enzyme produced in a 5-L stirred bioreactor was separated from culture liquid by ultrafiltration and then partially purified. After that, the enzyme was biochemically characterized and applied in grape juice processing. Finally, antimicrobial, antioxidant, and genotoxic potential of the ultrafiltrate was determined.

2 Materials and methods

2.1 Chemicals

Locust bean gum (LBG) and spent coffee grounds (SCGs) were obtained from GKM Carob Processing Factory and Starbucks® Coffee, respectively. Konjac glucomannan (KGM), guar gum (GG), and yeast mannan (YM) were procured from Smart Chemistry Trade and Consulting Limited Company (İzmir/Turkey), Kalipso Chemistry Limited Company (Maltepe/İstanbul), and Sigma–Aldrich (Darmstadt, Germany), respectively. Microbiological media and buffer components were purchased from Merck (Darmstadt, Germany). Plastic and glass materials were procured from Isolab (Eschau, Germany). Solvents and reagents used to prepare polyacrylamide gels, agarose gels, and chemicals employed in applying DNS, DPPH, and Folin–Ciocalteu methods were purchased from Sigma–Aldrich (Darmstadt, Germany). PageRuler™ Unstained Protein Ladder was procured from Thermo Fisher Scientific (Waltham, MA). Grapes (*Vitis vinifera* Linné subsp. vinifera) were purchased from the local market (Migros).

2.2 Microorganisms

Bacillus mojavensis TH309 was used as an extracellular endo-β-mannanase (BmMan) producer [15]. It was maintained on nutrient agar medium slants at 4 °C. The strain was grown at 45 °C with agitation at 200 rpm for 24 h in Luria–Bertani (LB) Broth. At the end of the incubation, cells were retrieved by centrifugation at 10 000 × *g* for 10 min and then diluted with distilled water to adjust to a turbidity equivalent of 0.5 McFarland Standard. The obtained suspension was re-centrifuged at 10 000 × *g* for 10 min, and then, cells were used as inoculum.

2.3 Optimization of media components

All experiments were carried out using 250-mL Erlenmeyer flasks containing 50 mL medium. The initial pH of the medium was adjusted to 7.5 with 5 M HCl or 5 M NaOH. Approximately 1×10^6 *B. mojavensis* cells were suspended in medium sterilized with an autoclave (Tuttnauer, 5050 ELV) at 121 °C for 15 min. Thereafter, the flasks were incubated for 72 h at 45 °C on an orbital shaker (Miprolab, Mci-120) with agitation at 200 rpm. At the end of incubation, a cell-free culture liquid was used as a crude enzyme.

2.3.1 Identifying the significant media components in mannanase production using Plackett–Burman design (PBD)

A total of ten factors including SCGs (A), yeast extract (B), peptone (C), meat extract (D), ammonium sulfate (E), KH_2PO_4 (G), $CaCl_2$ (H), K_2HPO_4 (J), NaCl (K), and $MgSO_4.7H_2O$ (L) were screened using PBD to identify the significant media components for mannanase production by submerged fermentation. Each media component was tested at low (-1) and high (+ 1) levels by performing 12 experimental runs designed based on a first-order polynomial linear model using Design Expert 7.0 software (Stat Ease Inc., Minneapolis, MN) (Supplementary information 1 and 2). Experiments were performed in triplicates, and results were expressed as mean mannanase activities. Based on ANOVA results, the media components with confidence levels of more than 95% ($p < 0.05$) were considered to have essential effects on mannanase production.

2.3.2 Response surface methodology–Box–Behnken design (RSM–BBD)

RSM–BBD has been applied to optimize the levels of significant media components, which were decided according to the previous selection study performed using PBD, and to evaluate their simultaneous interaction on mannanase production. Significant media components were assessed at three levels (Supplementary information 3) through RSM–BBD. According to the design, a total of 17 experiments were conducted in triplicates. The response value for each experiment was the mean value of mannanase activity of the crude enzyme. Building of a matrix for the design, fitting of experimental data to a second polynomial equation, statistical analysis, and the construction of three-dimensional (3D) response surface plot were done using the "Design Expert 7" software.

2.4 Submerged fermentation (SmF) in 5-L stirred bioreactor

SmF was performed in a 5-L stirred tank bioreactor (ElektroLab, FerMac 360) with a working volume of 3.5 L media optimized statistically by RSM–BBD. Incubation was carried out at 45 °C for 72 h without pH control and at an agitation speed of 200 rpm and aeration rate of 1vvm (volume per minute) after approximately 1×10^6 cells were inoculated. Twenty mL of culture was withdrawn at 12-h intervals to monitor the total protein concentration and BmMan yield, viable cell, endospore count, and pH of the medium.

2.4.1 Determination of viable cell and endospore counts

For determination of viable cell, tenfold serial dilutions of *B. mojavensis* TH309 culture were prepared in ¼ Ringer solution. Dilutions (100 µL) were spread on nutrient agar medium. Plates were incubated at 45 °C for 24 h. The mean average viable cell number in 1 mL of culture was calculated and given as colony-forming unit (CFU). To determine the spore counts, dilutions were incubated at 80 °C for 20 min. At the end of incubation, 100 µL of aliquots in dilutions was spread on same medium [16]. The number of colonies formed on medium surface was counted after the plates were incubated at 45 °C for 24 h. The mean average endospore in 1 mL of culture was calculated and presented as endospore-forming unit (EFU).

2.5 Preparation of cell-free culture liquid

Culture liquid was centrifuged (Nüve, NF 800R) at $10{,}000 \times g$ for 10 min. The supernatant was reserved for mannanase assay and estimation of total protein concentration.

2.6 Mannanase assay and estimation of total protein concentration

The mannanase activity was assayed using LBG as substrate. The enzyme solution (50 µL) was added to the 2% LBG solution (150 µL) prepared in 50 mM phosphate buffer (pH 7.0). The reaction mixture was incubated for 10 min at 50 °C. Subsequently, the reaction was terminated by adding 400 µL 3,5-dinitrosalicylic acid (DNS) reagent [17]. The solution was incubated in a heating block (Aosheng, MK-20) at 100 °C for 5 min, and then, absorbance at 540 nm was measured using a Multiplate reader (Thermo Scientific, Multiskan Go). The reducing sugar liberated was estimated from a D-mannose standard curve. One enzyme unit (U) of endo-β-mannanase is the amount of enzymes which release 1 µmol of equivalent to 1 µmol D-mannose per minute under assay condition (µmol/mL/min). Extracellular protein concentration was estimated by the method of Bradford [18] using bovine serum albumin (BSA) as standard.

2.7 Partial purification

Cell-free culture liquid recovered after SmF was passed through a 10-kDa molecular weight cutoff (MWCO) membrane using the Amicon™—Stirred Ultrafiltration Cells System (Merck KGaA, Darmstadt, Germany). Afterward, 20-fold concentrated proteins were subjected to the precipitation with ammonium sulfate at 70% of saturation level at

4 °C to partial purification [19]. Precipitated proteins were re-suspended in 50 mM phosphate buffer (pH 7) and then dialyzed against the same buffer at 4 °C. Finally, the partially purified BmMan was concentrated to 100-fold for further characterization studies.

2.8 Characterization of BmMan

2.8.1 Estimation of molecular weight of BmMan

Proteins in concentrated cell-free culture liquid were separated in 10% polyacrylamide gels containing 0.1% (w/v) LBG through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [20]. Electrophoresis was performed at 80 V using Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad Laboratories, Hercules, CA) under denaturing conditions [15]. Protein bands in a gel were visualized by staining with Coomassie brilliant blue R250. The protein band exhibits mannanase activity, which was determined by zymogram analysis. In the zymogram analysis, the other gel was first rinsed gently with 25% (v/v) isopropanol to remove SDS. Afterward, the gel was washed four times in 100 mM Tris–HCl buffer (pH 6.8) at 4 °C for 30 min. The enzyme was activated with incubation of the gel in the same buffer at 55 °C for 45 min. The gel was then stained with 0.1% (w/v) Congo red solution for 15 min. Finally, the gel was washed with 1 M NaCl solution for 4 h. Mannanase activity confirmed the presence of a light yellow band on the dark red polyacrylamide gel.

2.8.2 Effects of pH and temperature on BmMan activity and stability

The effect of pH on BmMan activity was assessed by conducting enzyme activity in various buffer systems (50 mM) at different pH values ranging from 3 to 10. The buffer systems used for adjusting the pH value of the reaction mixture were citrate (pH 3–6), phosphate (pH 6–8), and Tris–HCl (pH 8–10). The optimum temperature of BmMan was determined at optimal pH, varying the assay temperature from 20 °C to 90 °C. Maximum BmMan activity was accepted as 100%.

The influence of pH on BmMan stability was assessed by pre-incubation of enzyme in citrate buffer (50 mM) with various pH values (pH 3–6) at optimal temperature for 1 h and the subsequential calculation of residual activity. Buffer was exchanged using a centrifugal ultrafiltration device (Amicon Ultra-0.5, UFC5010, Merck). The thermal stability of BmMan was studied by pre-incubating the enzyme at 60–90 °C for 6 h and following the determination of residual activity under optimal conditions. BmMan activity before pre-incubation was considered as 100%.

2.8.3 Effects of metal ions and chemicals on BmMan activity

BmMan activity was individually assessed in the presence of metal ions (Ag^{2+}, K^{+}, Ca^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, and Co^{2+}) and chemicals (phenylmethylsulfonyl fluoride: PMSF, 1,4-dithiothreitol: DTT, Tween 20, Tween 80, Triton X-100, sodium dodecyl sulfate: SDS, β-mercaptoethanol: βME, and ethylenedinitrilotetraacetic acid: EDTA) at a final concentration of 1 mM or 10 mM (w/v). All assays were carried out under optimum conditions. BmMan activity in the absence of additives was accepted as 100%.

2.8.4 Effects of solvents on BmMan activity and stability

The effects of organic solvents were investigated by assaying BmMan activity in reaction mixtures supplemented with ethanol, acetone, methanol, dimethyl sulfoxide (DMSO), chloroform, isopropanol, and butanol at a final concentration of 10% (v/v). The activity of BmMan in the reaction mixture without solvent was assumed as 100%. The effect of solvents on the stability of BmMan was investigated by pre-incubation of the enzyme with solvents (10%) for 1 h at room temperature before enzymatic assay. Relative activity (%) was calculated by comparing the activity of solvent-free enzyme solutions pre-incubated under the same conditions (100%). Assays were performed under optimum conditions.

2.8.5 Substrate specificity of BmMan

The substrate specificity of BmMan (1 mg/mL) was assessed by assaying the enzyme activity against LBG, KGM, GG, and YM using the standard assay method under optimum conditions. Maximum activity was accepted as 100%.

2.9 Applicability of BmMan in grape juice processing

Briefly, 27 mL of crude grape juice extracted with a household electric juicer (Beko, BKK 3080 Rhb) was incubated with 1.5 mL of partially purified enzyme solution (30 U BmMan) at different temperatures (50–70 °C) for 60 min under 120 rpm shaking conditions. Subsequently, the mixture was centrifuged at 10,000 × g for 10 min to recover grape juice. Positive and negative control experiments were performed with the same procedure, using a commercial enzyme solution (30 U) and distilled water, respectively, instead of the partially purified enzyme solution. The commercial enzyme solution was prepared with pectinase from *A. niger* (Tito PK58, Smart Chemistry Trade

and Consulting Limited Company, İzmir/Turkey) due to its widespread use in fruit juice processing. The clarity of the resultant fruit juice was quantified by measuring the absorbance at 660 nm using a Multiplate reader. Reducing sugar content (RSC) and phenolic content (PC) were determined using DNS [17] and Folin–Ciocalteu method [21], respectively. Clarity, reducing sugar and phenolic content of negative control was accepted as 100%.

2.10 Biological properties of ultrafiltrate

Ultrafiltrate was dried using an oven to study its biological properties. Antimicrobial activity of ultrafiltrate against a Gram-negative bacterium *E. coli*, a Gram-positive bacterium *B. megaterium,* and a Gram-negative biofilm-forming bacterium *Pseudomonas putida* was evaluated by well diffusion test. Ultrafiltrate solution (50–200 µg/mL) was poured into wells (6 mm) cut out of the Luria–Bertani (LB) agar plate after fresh bacterial suspension (OD 0.5) was spread on medium surface. Subsequently, plate was incubated at 37 °C for 24 h. The antioxidant capacity of the dried ultrafiltrate was studied with of DPPH (2,2-diphenyl-1-picrylhydrazyl) method according to Könen-Adıgüzel et al. [22]. The genotoxic effect of ultrafiltrate was evaluated using comet assay. Experiments were carried out using the HepG2 cell line as mentioned by Yabalak et al. [23].

2.11 Statistical analysis

Results for each experiment, in triplicate, were expressed as the mean values. Microsoft Excel Software was used to calculate standard deviations and standard errors. Statistical significance of results was assessed by one-way ANOVA test computed with XL Toolbox NG (https://www.xltoolbox.net/) software.

3 RESULTS and DISCUSSION

3.1 Identification of significant media components in mannanase production using PBD

The PDB is a powerful tool which was used to screen the effects of media components on BmMan production. The wide variation observed in experimental results (Supplementary information 2) reveals the importance of the media components in BmMan production. The quality of the model was checked by analysis of variance (ANOVA). Accordingly, the model was statistically significant with F-value of 1220.12 and p-value of 0.0223. Furthermore, it was verified with the 0.9999 of determination coefficient (R^2), 0.9991 of adjusted determination coefficient (Adj-R^2) and 0.9882 of predicted determination coefficient (Pred-R^2) values. Based on the experimental results, the first-order polynomial equation was obtained as follows:

$$\begin{aligned} BmManproduction\left(\frac{U}{mL}\right) : {} & 7.39262 + 0.23032SCG \\ & + 0.11578yeastextract - 0.4922peptone \\ & - 0.45722meatextract \\ & - 0.46165ammoniumsulfate \\ & - 0.57061KH_2PO_4 + 2.01865CaCl_2 \\ & - 0.53574K_2HPO_4 - 0.46645NaCl \\ & + 2.63453MgSO_4.7H_2O. \end{aligned}$$

The main effect of each media component on the yield was shown in the Pareto chart (Fig. 1). SCG, $CaCl_2$, and $MgSO_4$ were significant media components with a p-value of 0.0166, 0.0189, and 0.0145, respectively. Yeast extract has a certain positive effect with a p-value of 0.0549 in the BmMan production, but its contribution is not statistically significant.

On the other hand, it was observed that peptone, meat extract, ammonium sulfate, KH_2PO_4, K_2HPO_4, and NaCl affected BmMan production negatively. Therefore, media components with a negative effect were removed in media formulation, while yeast extract was kept a minimum level. SCG, $CaCl_2$, and $MgSO_4$ were selected for further RSM–BBD study.

3.2 RSM–BBD

RSM–BBD was applied to determine the optimal combination of SCG (X_1), $CaCl_2$ (X_2), and $MgSO_4$ (X_3) levels and their interactive effect on BmMan production. The experimental results with a large fluctuation range from 35.65 U/mL (run 16) to 3.95 U/mL (run 11) depicted that the optimization of the medium components is the preferable alternative for the overproduction of BmMan. The fitness of the model was verified using some statistical parameters. Among them, p-value of < 0.0001 indicated that the model was highly significant. F-value of 98.96 implied the model showing significance. R^2-value of 0.9922 indicated that the experimental results were in fair compatible with predicted results. Further, Adj-R2 and Pred-R^2 were 0.9822 and 0.9059, respectively. Lack of fit was not significant with a value of 0.1260. These findings depicted the goodness of the predictive ability of the suggested model. The experimental results shown in supplementary information 4 were fitted into the following second-order polynomial equation to predict BmMan production.

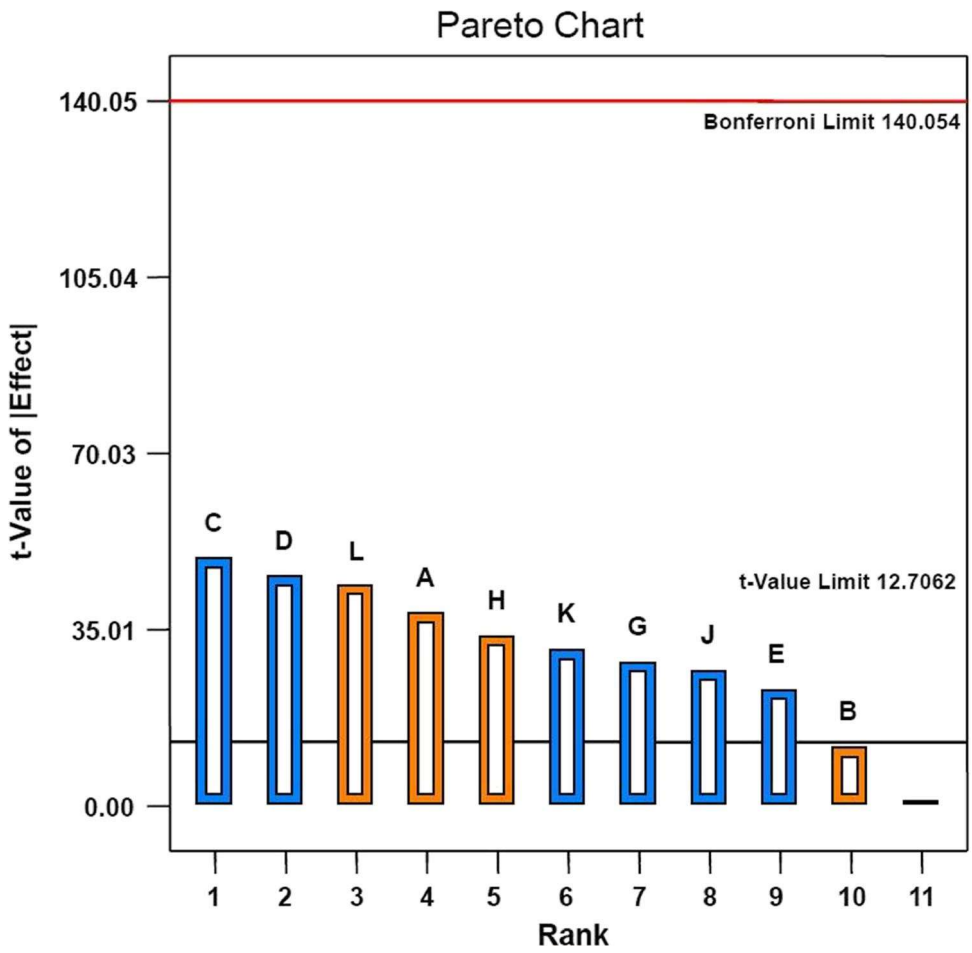


Fig. 1 The Pareto chart shows the order and significance of the factors (A: SCW, B: yeast extract, C: peptone, D: meet extract, E: ammonium sulfate, G: K_2HPO_4, H: $CaCl_2$, J: K_2HPO_4, K: NaCl, L: $MgSO_4.7H_2O$) influencing the mannanase production by *B. mojavensis* TH309. Blue and orange bars reflect negative and positive effects, respectively

$$BmManproduction\left(\frac{U}{\text{mL}}\right) : -199.55843 + 22.46674X_1, +33.19507X_2 + 37.19507X_3 - 0.48300X_1X_2 - 0.57450X_1X_3 - 5.75465X_2X_3 - 0.70318X_1^2 - 3.34243X_2^2 - 4.80492X_3^2.$$

Furthermore, analysis of variance shown in supplementary information 5 denoted that linear, quadratic, and interaction model terms are significant with *p*-values less than 0.05, statistically. Both cross-product model term X_2X_3 and quadratic model term X_1^2 exhibited the highest effect with a p-value less than 0.0001, followed by X_1($p < 0.0002$).

Individual and interactive effects of any two media components on BmMan production were evaluated through 3D response surface plot. During the evaluation, the concentration of the other component was kept at the middle value. Figure 2a reveals that the BmMan production was notably enhanced with increasing SCG concentration up to its middle level. On the other hand, a gradual increase in BmMan titer was observed with the increase in $CaCl_2$ concentration at all levels of SCG. According to Fig. 2b, a similar convex trend was monitored involving SCG and $MgSO_4$. The highest enzyme titer was detected at the middle levels of SCG and $MgSO_4$. Interaction between SCG and $MgSO_4$ also revealed that at lower and higher concentrations, both did not favor BmMan production. $MgSO_4$ enhanced BmMan production at both low and middle levels of SCG. Figure 2c depicts the interaction of $CaCl_2$ and $MgSO_4$ on BmMan production. The increase in $CaCl_2$ resulted in the elevation of BmMan production when $MgSO_4$ concentration adjusted to less than its middle level. A linear increase in BmMan production was observed with the elevation of $CaCl_2$ up to the middle level. Thereafter, the production rate was decreased gradually. $MgSO_4$ induced enzyme production at only low level of $CaCl_2$. On the other hand, the enzyme production dramatically diminished with increasing $MgSO_4$ concentration as the $CaCl_2$ kept at a high level.

According to the numerical optimization performed by Derringer's desirability function [15, 24], the optimal concentrations of SCG, $CaCl_2$, and $MgSO_4$ are found to be 14.46 g/L, 2.92 g/L, and 1.26 g/L, respectively. Maximum BmMan yield is predicted to be 35.55 U/mL with a desirability value of 0.997 in the optimized fermentation medium.

BmMan was produced in 50 mL fermentation medium (pH 7.5), prepared with the aforementioned concentration of each variable and 1 g/L yeast extract to validate the predicted result. Experiments were carried out three times in a 250-mL

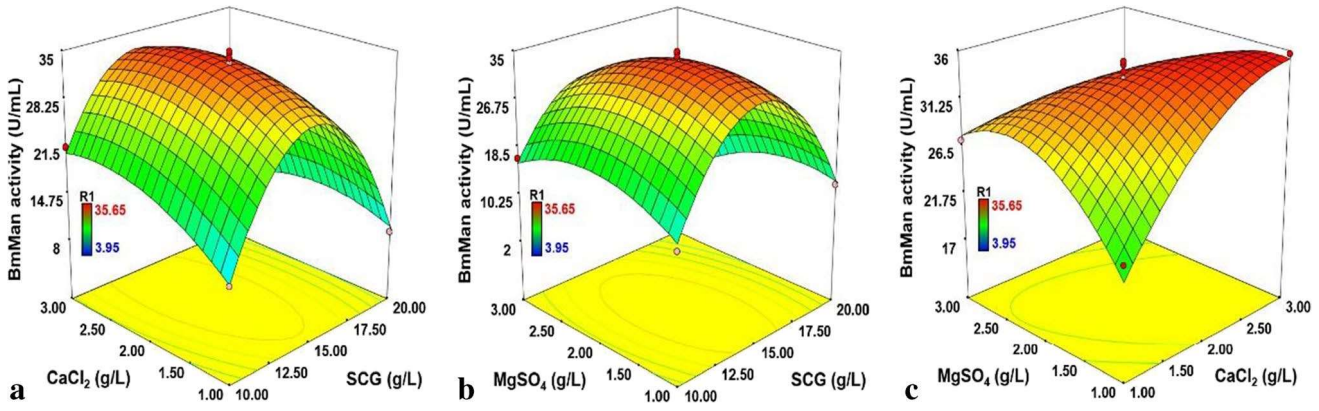


Fig. 2 The RSM plots illustrating the combinational effects of **a** $CaCl_2$ and SCG, **b** $MgSO_4$ and SCG, and **c** $MgSO_4$ and $CaCl_2$ on mannanase production by *B. mojavensis*. Experiments were carried out at 45 °C for 72 h under 200 rpm shaking conditions

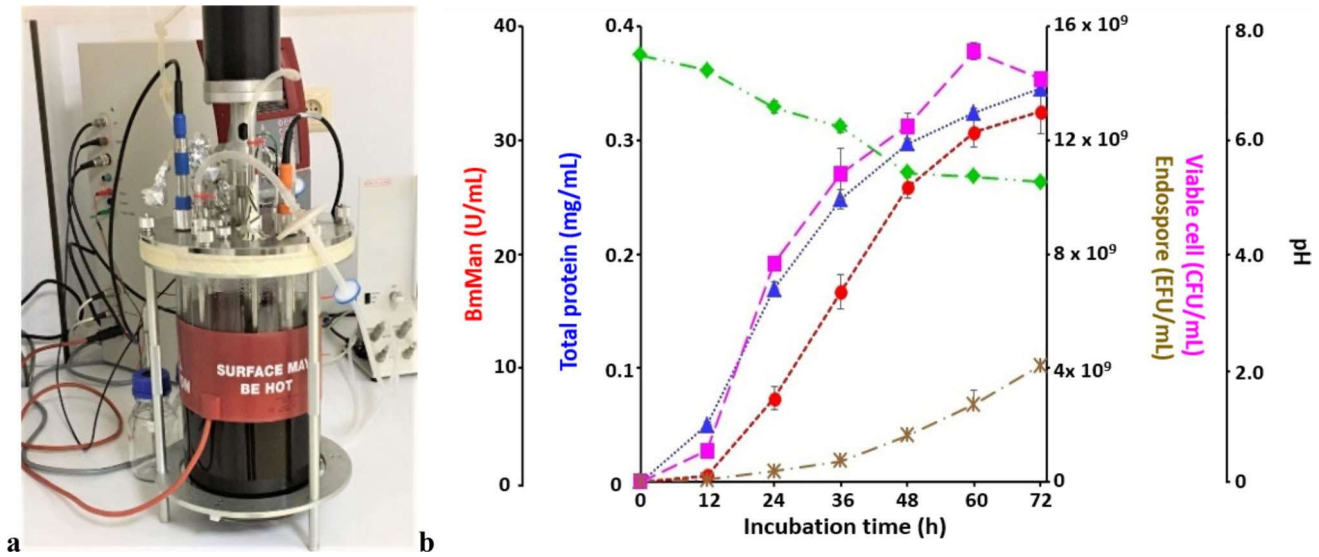


Fig. 3 **a** Fermentation of *B. mojavensis* in 5-L stirred bioreactor containing optimized medium. **b** Time course of BmMan titer (red, filled circles), total protein (blue, filled triangles), endospore count (brown, crosses), viable cells (purple, filled squares), and pH (green, filled diamonds) during fermentation

Erlenmeyer flask for 72 h at 45 °C under 200 rpm shaking conditions. The experimental BmMan yield (34.80 ± 1.44), which was in high agreement with the predicted result, confirmed the validity.

3.3 BmMan production in 5-L stirred bioreactor

Time course of BmMan yield, total protein, bacterial growth, endospore formation, and pH value of the medium during the incubation of *B. mojavensis* TH309 in a 5-L stirred bioreactor with the optimized medium (Fig. 3). BmMan titer increased significantly from 0.53 U/mL to 30.74 U/mL between the 12th h and 60th h of incubation. BmMan titer reached to 32.55 U/mL at the end of incubation. A similar trend was observed for total protein concentration. Viable cell increased to the maximum value (15.13×10^9 CFU) at 60th h and decreased (14.16×10^9 CFU) thereafter. A significant endospore formation was monitored at 24th h (0.36×10^9 EFU) and then gradually elevated (4.08×10^9 EFU). The pH of the medium decreased from an initial value of 7.5 to 5.0 at the end of SmF.

3.4 Partial purification of BmMan

Purification steps of BmMan are summarized in supplementary information 6. Cell-free culture liquid containing 104,150.40 U BmMan and 1106.28 mg protein was first subjected to ultrafiltration. The remaining BmMan activity

and protein were 86,529.60 U and 896.00 mg, respectively. After that, precipitation with ammonium sulfate resulted in 2.20-fold purification with 67.31% yield compared with cell-free culture liquid. Following the dialysis of precipitated proteins, the final purification fold and yield were 2.28 and 63.01%, respectively.

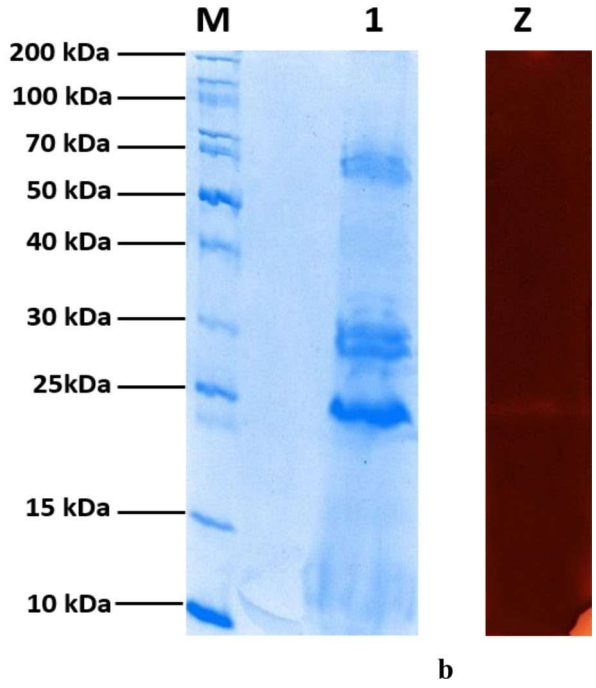


Fig. 4 **a** SDS–PAGE analysis of partially purified BmMan. Lane M and Lane 1 indicate protein ladder and partially purified BmMan, respectively. **b** Zymogram analysis of partially purified BmMan

3.5 Characterization of BmMan

3.5.1 The molecular weight of BmMan

The combined use of SDS–PAGE and zymography allows to quickly determine the molecular weight of a specific enzyme without purification. Position of proteins in the partially purified BmMan was monitored by SDS–PAGE (Fig. 4a). The protein band corresponding to mannanase activity was detected by zymography (Fig. 4b). Accordingly, it was seen that the molecular weight of BmMan was approximately 24 kDa, which was lower than those of mannanases from many bacterial and fungal strains [25–30]. This suggests that BmMan is one of the few documented low molecular weights mannanase [31, 32]. Comparison of molecular weight of mannanases from B. mojavensis and other microbial sources is shown in supplementary information 7. Results also showed that BmMan was a monomeric protein.

3.5.2 Effects of pH and temperature on BmMan activity and stability

The effect of pH on BmMan activity is shown in Fig. 5a. BmMan was most active at a pH range from 3 to 7 and exhibited maximal activity at pH 4. BmMan maintained more than 75% of its maximum activity at pH 7. However, the activity of the enzyme decreased sharply above pH 7. Interestingly, the acidophilic characteristic of BmMan is unlike its counterparts from other *Bacillus* species. It was reported that mannanases from *Bacillus* sp. N16-5 [33], *Bacillus halodurans* PPKS-2 [34], *Bacillus nealsonii* PN-11 [20], *B. subtilis* subsp. *inaquosorum* CSB31 [35], *Bacillus clausii* S10 [36], and *Bacillus pumilus* M27 [37] showed maximum activity at alkaline pHs. On the other hand, optimal pH has been detected to be 7.0 for mannanases from

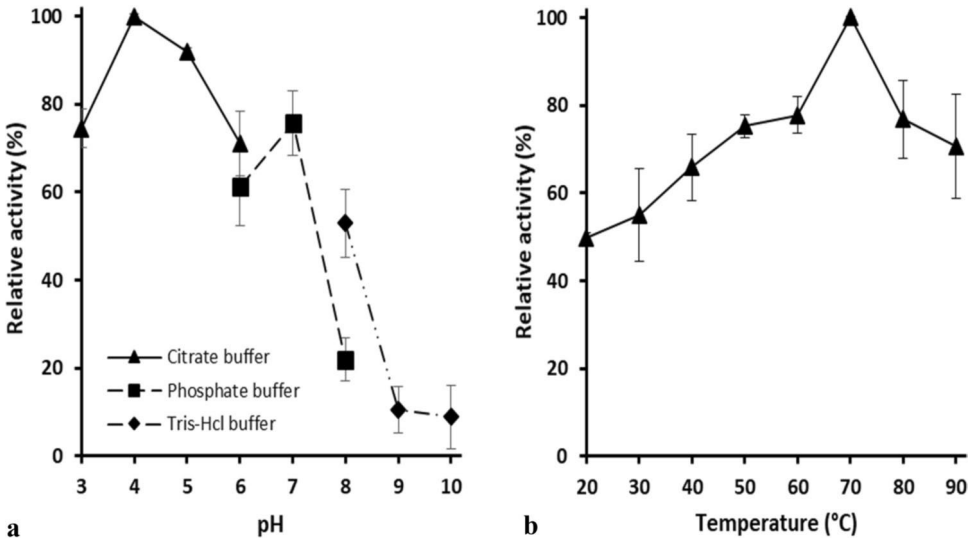


Fig. 5 **a** The effect of pH on BmMan activity. Citrate (filled triangles), phosphate (filled squares), and Tris–HCl (filled diamonds) buffers (50 mM) were used to adjust pHs. Assays were performed at 50 °C. **b** The effect of temperature on BmMan activity. The reaction mixture was prepared with 50 mM citrate buffer (pH 4). The maximum BmMan activity was accepted as 100% for each set of experiments. Data were presented with standard error bars

Bacillus velezensis BS-37 [38], *Bacillus* sp. KW1 [26], and *Bacillus licheniformis* [39]. Mannanases from *Bacillus subtilis* MAFIC-S11 [40], and *Bacillus circulans* NT 6.7 [41] have also exhibited a maximal activity at pH 6.

As shown in Fig. 5b, BmMan was active at all tested temperature ranges (20 °C—90 °C). The apparent optimum temperature of BmMan was 70 °C. It also retained 76.8% and 70.6% of its maximum activity at 80 °C and 90 °C, relatively. In addition, a gradual reduction in enzyme activity was observed with decreasing temperature. However, the relative activity of BmMan was still higher than 45% at 20 °C. The optimum temperature of BmMan was higher than those of acidophilic mannanases from *Providencia vermicola* [42], *Penicillium citrinum* [43], and *Microbacterium* sp. CIAB417 [9]. A similar optimal temperature has been reported for acidophilic mannanase from *Aspergillus calidoustus* [29]. Liu et al. [27] reported that the recombinant mannanase from *Aspergillus kawachii* IFO 4308 is also 80 °C. In fact, there are a few studies in the literature describing the thermal properties of acidophilic mannanase from bacteria. However, many reports available involve thermophilic or thermotolerant bacterial mannanases. Among them, an optimum temperature similar to that of BmMan has been recorded for mannanases from *Klebsiella pneumonia SS11* [44] and *Bacillus subtilis* US191 [45], while mannanase from *Rhodothermus marinus* [46] and *Thermotoga petrophila* [47] has been reported to have an optimum temperature higher than that of BmMan, respectively.

The influence of pH on BmMan stability is shown in Fig. 6a. BmMan was stable at acidic pH values (pH 3–6) and showed better stability at pH 4 with 93.3% relative activity. Besides, the enzyme retained more than 70% of its catalytic activity at pH 7. On the other hand, the relative activity of BmMan diminished significantly above pH 8.0 and fell to less than half of the initial. Accordingly, the results showed that BmMan, which is both active and stable in the acidic environment, is more attractive than its counterparts for use in the feed industry. It has been reported that β-mannanase from *Bacillus subtilis* [48] and *Bacillus pumilus* GBSW19 [49] was stable after pre-incubation at pH 5. Similarly, Liu et al. [50] showed that β-mannanase from *Bacillus subtilis* YH12 maintained more than 80% of its activity in 50 mM citrate buffer with a 4.5 pH value. The thermal stability study revealed that BmMan was quite stable at 50–70 °C (Fig. 6b). However, BmMan lost about 75% of its activity after pre-incubation at 80 °C. The half-life ($t_{1/2}$) of BmMan was calculated to be 40.8, 150.2, 210.2, and 387.6 min at 80, 70, 60, and 50 °C, respectively. Although some mannanases with thermostable properties have been reported previously, only a few are more stable than BmMan at 70 °C [26, 34]. In addition, temperature and pH characteristics of BmMan and its counterparts are detailed compared in supplementary information 8. Results displayed that BmMan could take further advantage in harsh industrial applications performed under acidic conditions.

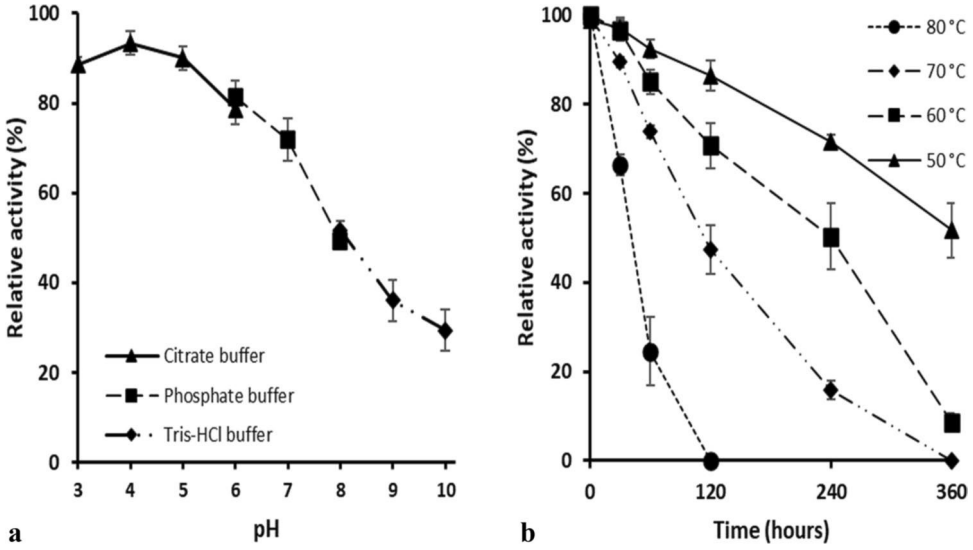


Fig. 6 **a** The effect of pH on BmMan stability. BmMan was pre-incubated at different pHs before being assayed. Pre-incubation was performed at 50 °C. Citrate (filled triangles), phosphate (filled squares), and Tris–HCl (filled diamonds) buffers (50 mM) were used to pH 3–6, pH 6–8, and pH 8–10, respectively. **b** The effect of temperature on BmMan stability. The enzyme was pre-incubated at 50 °C (filled triangles), 60 °C (filled squares), 70 °C (filled diamonds), and 80 °C (filled circles) for 0–360 min. Initial BmMan activity was accepted as 100% for each set of experiments. Data were presented with standard error bars

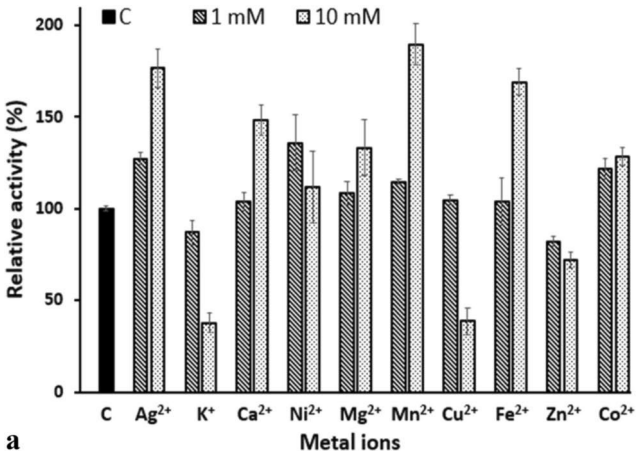
3.5.3 Effects of metal ions and chemicals on BmMan activity

As depicted in Fig. 7a, Ag^{2+} (1 and 10 mM), Ca^{2+} (10 mM), Ni^{2+} (1 mM), Mg^{2+} (10 mM), Mn^{2+} (10 mM), Fe^{2+}, and Co^{2+} (1 and 10 mM) enhanced BmMan activity, significantly. Similarly, it has been reported that Ca^{2+}, Co^{2+}, and Mn^{2+} marginally activated the activity of mannanases from *Bacillus subtilis* B36 [51] (Li 2006), *Cellulosimicrobium* sp. HY-13 [52, 53], *Bacillus subtilis* MAFIC-S11 [40], *Streptomyces* sp. CS147 [32], *Pediococcus acidilactici* [54], *Lichtheimia ramosa* [55], and *Paenibacillus* sp. CH-3 [6]. Improvement in mannanase activity in the presence of Ni^{2+}has also been demonstrated by Chauhan et al. [20]. Although Ag^{2+} mostly inhibits the activity of mannanases, it was observed to increase BmMan activity up to 163.7%. It may be due to more sulfhydryl groups than others in BmMan [56]. In contrast to our findings, Mg^{+2} has been reported to be a strong inhibitor for mannanases from *Penicillium italicum* [57], *Streptomyces* sp. CS147 [32], and *Streptomyces thermolilacinus* [58]. BmMan was inhibited with K^{+} (10 Mm) and Cu^{2+} similar to mannanases from *Paenibacillus* sp. CH-3 [6] and *Thermoanaerobacterium aotearoense* SCUT27 [8].

The influence of various chemicals on BmMan activity is illustrated in Fig. 7b. The addition of DTT and βME in the reaction mixture increased the BmMan activity, indicating that the enzyme contains free sulfhydryl groups of free cysteine residues. Similar behavior has been reported for mannanases from *Streptomyces* sp. Alg-S25 [59], *Bacillus pumilus* Nsic-2 [60], and *Verrucomicrobiae* sp. DG1235 [61]. BmMan was also stimulated with a non-ionic surfactant Triton X-100. Tween 20 had little influence on the enzyme. BmMan activity decreased significantly in the presence of PMSF and Tween 80. In addition, a strong effect of EDTA on the enzyme activity was also detected, and it suggests that BmMan is a metalloenzyme that needs metal ions for activity.

3.5.4 Effect of solvents on BmMan activity and stability

Ethanol, acetone chloroform, and isopropanol markedly improved BmMan activity up to 133.5%, 149.4%, 142.0%, and 156.5%, respectively. In the presence of DMSO (115.2%) and butanol (114.3%), a moderate increase in enzyme activity was recorded (Fig. 8). Similar effects of these solvents were also observed on mannanase from *Bacillus subtilis* subsp. inaquosorum CSB31 [35]. It has also been reported that the activities of ManAo, a thermophilic and acidic β-mannanase, were enhanced in the presence of chloroform, acetone, and methanol [56]. On the other hand, a sharp decrease in the relative activity was also observed by pre-incubating BmMan with ethanol (69.4%) and acetone (65.3%). In addition, BmMan activity decreased to 52.4%, 80.3%, and 71.5% when the enzyme was pre-incubated with chloroform, isopropanol, and butanol at a final concentration of 10%, respectively. Similar findings have been reported by Lima et al. [43]. Aprotic organic solvents like DMSO could keep BmMan kinetically trapped in its active conformation. Thus, they could allow the enzyme to remain catalytically active for long periods. On the other hand, the low stability of BmMan against ethanol and acetone could be due to structural perturbations or partial denaturation resulting from water removal or solvent insertion.



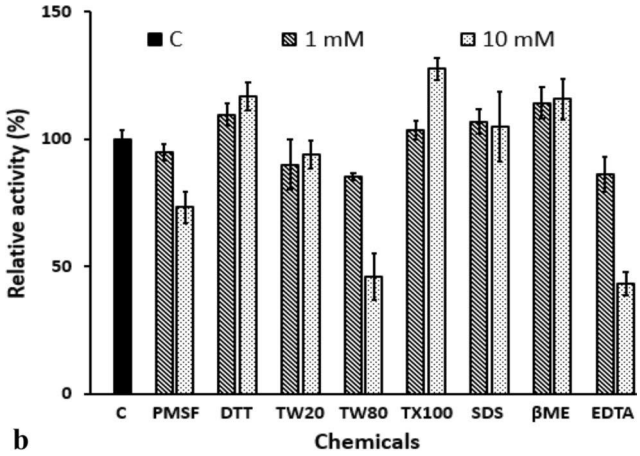


Fig. 7 Effects of metal ions **a** and chemicals **b** on BmMan activity. The activity of BmMan was assayed in the presence of 1 mM (filled diagonal lines) or 10 mM (filled points) additives under optimum conditions. BmMan activity in the absence of additive was accepted as control (100%). Results of experiments in triplicates were presented with standard error bars

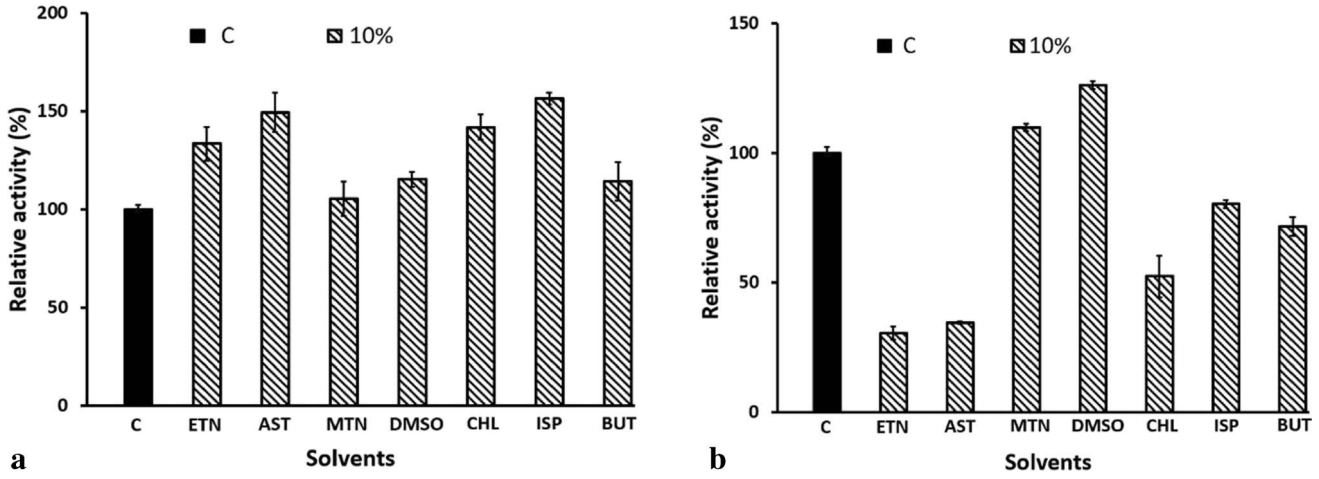


Fig. 8 **a** The effect of solvents on BmMan activity. The activity of BmMan in the reaction mixture without solvents was accepted as a control (100%). **b** The effect of solvents on BmMan stability. Initial BmMan activity was accepted as control (100%). Each set of experiments was performed in triplicates and presented with standard error bars

3.5.5 Substrate specificity of BmMan

Among the substrates tested, BmMan exhibited maximum hydrolytic activity toward LBG (100%). The relative activity of BmMan toward KGM and GM was around 86.4% and 38.8, respectively. Moreover, the activity of BmMan against YM was the lowest, with a relative activity of 14.3%. Results showed that the BmMan prefers the mannans with a low level of α-(1–6)-D-galactose branching. Similarly, Malgas et al. [5] demonstrated that ManA from *C. cellulovorans* and Man26A from *A. niger* exhibited high activity on locust bean gum (100% relative activity), but considerably lower activity on guar gum (approximately 50% relative activity). The extremely low activity of BmMan toward YM is likely due to the enzymes poor specificity for α-glycosidic linkages found in YM [62].

3.6 Applicability of BmMan in grape juice processing

Treatment of grape juice with BmMan improved clarity, reducing sugar content (RSC), and phenolic content (PC) at all tested temperatures (Table 1). The maximum improvement was recorded at 70 °C, which is the optimum temperature for BmMan. At this temperature, increased clarity, RSC, and PC were 82.6%, 61.5%, and 80.7%, respectively, compared with the negative control. In contrast, the commercial enzyme was not as successful as BmMan in improving juice quality, even at optimum operating temperature (50 °C). Earlier studies denoted that the treatment with mannanases positively contributes to fruit juices' physical and nutritional quality due to the catalytic effect of mannanases on soluble mannans and hemicellulolytic mannans in fruits [2, 63]. However, there are a few reports that emphasize enhancing the grape juice quality. Previously, it has been reported that the mannanase from *Pediococcus acidilactici* increased clarity and reducing sugar content of grape juice from 72.23% to 84.65% and from 3.27 mg/mL to 5.85 mg/mL, respectively [54]. In another study, only the yield-enhancing effect of mannanase in the grape juice process has been shown [64].

Table 1 Percent increase in clarity, RSC, and PC of grape juice with enzymatic treatment for 60 min at different temperatures. Experiments were carried out in triplicate and expressed with standard deviation

Temperature	Enzyme	Clarity	RSC	PC
		(Increase %)		
50 °C	Commercial enzyme	66.1 ± 2.3	54.6 ± 6.3	20.8 ± 2.9
	BmMan	30.4 ± 1.5	60.0 ± 4,1	22.9 ± 3.0
60 °C	Commercial enzyme	56.4 ± 3.6	52.8 ± 4.3	19.0 ± 2.4
	BmMan	46.7 ± 2.7	81.9 ± 3.8	29.6 ± 2.7
70 °C	Commercial enzyme	15.4 ± 2.9	40.8 ± 3.6	5.10 ± 0.5
	BmMan	82.6 ± 3.1	80.7 ± 4.1	61.5 ± 2.6

RSC: Reducing sugar content

PC: phenolic content

3.7 Biological properties of ultrafiltrate

Antimicrobial activity of ultrafiltrate was assayed using well diffusion test. The presence of clear zones around wells in agar plates is considered the result of the antimicrobial activity of substances. Considering the results in this context, the dried ultrafiltrate showed excellent antimicrobial

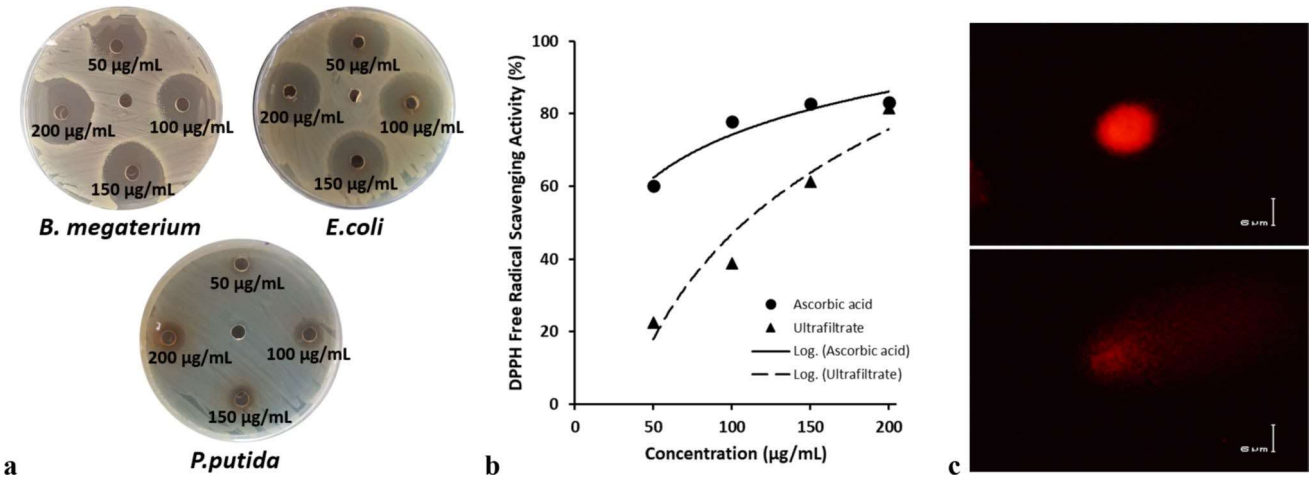


Fig. 9 **a** Well diffusion assay. **b** DPPH free radical scavenging activity of dried ultrafiltrate at different concentrations. Ascorbic acid used as positive control. **c** Undamaged and damaged nuclei detected with the comet assay

activity against *B. megaterium* and *E. coli*. In contrast, moderate antimicrobial activity was recorded against *P. putida* (Fig. 9a). The less inhibitory effect of the ultrafiltrate against *P. putida* than the others may be due to their broad metabolic versatility and different mechanisms, which allows them to adapt to different habitats and nutritional environments. Among them, biofilm formation could be limited the interaction between the ultrafiltrate and the cell. The antioxidant capacity of dried ultrafiltrate was assessed based on DPPH free radical scavenging activity. Results indicated that the dried ultrafiltrate had almost half the antioxidant capacity of ascorbic acid (positive control) (Fig. 9b). Genotoxicity of the dried ultrafiltrate was assessed with comet assay using HepG2 cell line, an in vitro model for routine genotoxicity studies [65, 66]. According to results (data not showed), the dried ultrafiltrate did not significantly induce DNA damage in HepG2.

4 Conclusions

Mannanase (BmMan) production by *B. mojavensis* TH309 in SmF using SCGs was enhanced ~ 32-fold by statistical optimization. Production with the optimized medium was successfully scaled up to a 5-L stirred bioreactor. Thus, a new approach that may decrease the cost and help solid waste management was provided for the commercial production of mannanase. BmMan was acidophilic and adequately thermostable for application in food and feed industries. Further characterization studies showed that the enzyme could endure harsh industrial conditions. The treatment with BmMan enhanced the reducing sugar, total phenolic content, and clarity of grape juice. In addition, it was demonstrated that waste products of the BmMan production process could be utilized as an antimicrobial and an antioxidant agent.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13399-022-02602-1.

Declarations

Competing interests The authors declare no competing interests.

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