EFFECT OF PH ON CONSTITUTIVE IMMOBILIZATION OF β-MANNANASE ON THE *LACTOBACILLUS* CELL SURFACE

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(Received: April 06, 2025; Revised: June 10, 2025; Accepted: June 19, 2025)

DOI: 10.31130/ud-jst.2025.23(9C).530E

Abstract - Previously, β-mannanase (ManB) was successfully immobilized on the surface of the *Lactobacillus plantarum* using two constitutive promoters, Pgm and SlpA. The highest activity of immobilized ManB (3010 U/g dry cell weight) was obtained after 4 hours of induction culture, extending cultivation to 8 hours led to a 4.2-fold decrease. This decline might be due to lactic acid production by *L. plantarum*, which lowered the pH of the culture medium. This study evaluated the impact of pH-controlled cultivation on the activity of ManB displayed on *L. plantarum* WCFS1. Under these conditions, the highest ManB activity decreased by only ~1.5-fold after 8 hours. This demonstrates that maintaining a constant pH level is a critical factor for improving bacterial cell growth and enhancing the overall yield of enzymes displayed *L. plantarum* surface. This is the first study to investivate the effect of pH on constitutive immobilization of ManB on the *Lactobacillus* cell surface.

Key words - Immobilization; mannanase; pH; *Lactobacillus plantarum*; constitutive promoter

1. Introduction

Lactobacillus plantarum is the lactic acid-producing bacterium widely recognized as a safe probiotic [1]. This Gram-positive bacterium, originally isolated from human saliva, has a thick cell wall structure and can metabolize various types of sugars. Notably, *L. plantarum* can survive in the stomach for up to seven days, demonstrating its strong adhesion capacity to the intestinal mucosa [2]. Therefore, many studies have employed *L. plantarum* as a microbial cell factory for production of enzymes toward converting low-cost agricultural waste into value-added bioactive compounds [3]. This approach not only helps mitigate environmental pollution from large quantities of agricutural waste, but also contributes to the development of health-promoting products.

β-mannanase (EC 3.2.1.78) is a hydrolytic enzyme that can break down 1,4-manosidic linkages in mannan, galactomannan into mannooligosaccharides (MOS) and small amounts of mannose, glucose and galactose [4]. Notably, MOS has been demonstrated to exert health-promoting effects on both humans and animals [5]. Thus, many research groups are focusing on the production of β-mannanase for the conversion of agricultural waste, such as copra meal into MOS chains. However, utilization of free enzymes requires complex purification and concentration processes, and more importantly, the free enzymes cannot be recovered after enzymatic reactions. To overcome these limitations, immobilizing β-mannanase on the bacterial surface by genetic engineering is an alternative strategy that enables reuse and simplifies downstream processing.

In the genetically engineered enzyme immobilization method, mannanase is fused with an anchoring motif and expressed in the probiotic bacterium *Lactobacillus plantarum*. During bacterial growth, the synthesis and surface display of the enzyme occur simultaneously [6]. A key advantage of this approach is that enzyme-carrying cells can be easily produced using simple microbial cultivation methods, and they offer easy recovery, high stability, reusability, and convenient storage.

In 2019, constitutive immobilization of ManB on *L. plantarum* WCFS1 cell surface was successfully developed by using Pgm and SlpA, two constitutive promoters derived from *L. acidophilus* NCFM's phosphoglycerate mutase (*pgm*) and *L. acidophilus* ATCC 4356's S-layer protein SlpA, respectively [7]. These strong promoters enabled the constitutive expression and immobilization of the enzymes without needing an inducer. This offers significant benefits for developing safe, food-grade whole-cell biocatalysts relevant for producing health-promoting oligosaccharides. The previous study showed that under pH-uncontrolled conditions, the highest activity of ManB immobilized on *Lactobacillus* cells driven by the SlpA promoter was 3010 U/g dry biomass after 4 hours of cultivation but dropped sharply (approximately 4.2-fold) after 8 hours [7]. This decline may have been caused by lactic acid production during *L. plantarum* fermentation, which lowered the culture medium's pH. It's hypothesized that the acidic pH either deactivated the surface-immobilized ManB or inhibited its expression and surface synthesis. Currently, no reports detail the role of pH in the constitutive expression and immobilization of enzymes on the bacterial cell surface. Therefore, the present study aims to determine the effect of pH on the growth of recombinant *Lactobacillus* bacteria and the activity of ManB displayed on the surface of *L. plantarum* WCFS1 harboring the Pgm and SlpA promoters.

2. Materials and methods

2.1. Microbial strain and culture conditions

Lactobacillus plantarum WCFS1 was originally isolated from human saliva, as described by Kleerebezem et al., and was obtained from NIZO Food Research (Ede, the Netherlands) [2]. The strain is currently maintained in the microbial culture collection of the Norwegian University of Life Sciences (Ås, Norway). *L. plantarum* was cultured in de Man, Rogosa and Sharpe (MRS) broth

(Oxoid) at 37°C without shaking. When required, erythromycin was added to the medium at a final concentration of 200 µg/ml. Agar plates were prepared by supplementing the broth medium with 1.5% (w/v) agar. All recombinant plasmids used in this study are listed in Table 1.

Table 1. *Plasmids used in this study*

Plasmid	Relevant characteristics
pEV	Erm^r; pLp 2578sAmyA derivative, inducible promoter, no signal sequence, no *man* (negative control) [9]
pSIP_1261ManB	Erm^r; pLp 1261Inv derivative, inducible promoter, lipoanchor sequence from Lp_1261 fused with fragment of *man-myc* instead of *inv* (positive control) [10]
pPgm_1261ManB	Erm^r; pSIP 1261ManB derivative with constitutive promoter *pgm* [7]
pSlpA_1261ManB	Erm^r; pSIP 1261ManB derivative with constitutive promoter *slpA* [7]

2.2. Gene expression in L. plantarum cells harboring different recombination plasmids

Overnight cultures of *Lactobacillus plantarum* WCFS1 harboring different recombinant plasmids (pEV, pSIP_1261ManB, pPgm_1261ManB, and pSlpA_1261ManB) were inoculated into 50 mL of MRS medium supplemented with erythromycin to an initial OD_{600} of approximately 0.1 (time point 0 h). For cells containing the plasmids pPgm_1261ManB and pSlpA_1261ManB, cell cultures were harvested after 4 hours of cultivation. In the case of induced expression (cells carrying pEV and pSIP_1261ManB), ManB surface display was induced by the addition of the synthetic pheromone IP-673 when the culture reached an OD_{600} of 0.3 (approximately 2 hours from time point 0 h). The cell cultures harboring pEV and pSIP_1261ManB were harvested 2 hours after induction.

To evaluate the effect of pH on the growth of recombinant *Lactobacillus* cells driven by the constitutive promoters Pgm and SlpA, overnight cultures of *L. plantarum* WCFS1 harboring pPgm_1261ManB, and pSlpA_1261ManB were inoculated into 50 mL of MRS medium supplemented with erythromycin to an initial OD_{600} of approximately 0.1 (time point 0 h). Culture aliquots were then taken at regular two-hour intervals to measure the optical density at 600 nm. Samples with $OD_{600} > 0.8$ were diluted to ensure readings were within the linear range.

2.3. Batch fermentation with pH control

Batch fermentation was carried out following method described previously [10]. *Lactobacillus plantarum* WCFS1 harboring the recombinant plasmids pPgm_1261ManB and pSlpA_1261ManB, which contain the gene encoding mannanase, was cultured in MRS medium supplemented with 5 µg/mL erythromycin. After 16–18 hours of cultivation, the seed culture was diluted into 50 mL of fresh medium at 37°C to an initial OD_{600} of ~0.1.

The batch fermentation with pH control was conducted in 400 mL medium using the HT-Multifors fermentation system (Infors HT, Switzerland). The culture pH was maintained at 6.0 using sodium hydroxide 1M, and agitation was set at 200 rpm. After 4 hours of cultivation, the culture broth was taken at regular intervals of one hour to measure optical density at 600 nm and to determine the catalytic activity of mannanase immobilized on the surface of *L. plantarum* cells.

2.4. Enzymatic activity assay

Enzymatic activity was determined following the method previously described [8, 9]. Basically, the reaction mixture consisted of 100 µL of a suspension of the enzyme-displaying cells in PBS and 900 µL of 0.5% (w/v) galactomannan solution (locust bean gum, LBG; Megazyme, Bray, Ireland). The substrate solution was prepared by dissolving LBG in 50 mM sodium citrate buffer (pH 6.0) at 50°C for 30 minutes.

After the initial 4-hour cultivation, cells were harvested by centrifugation at 4000 × g for 5 minutes at 4°C. The pellet was washed twice with PBS buffer and then resuspended in 100 µL of PBS. The reaction was initiated by incubating the cells with the substrate solution at 37°C, 600 rpm for 5 minutes. The reaction was terminated by centrifugation at 5000 × g, 4°C for 2 minutes. The amount of reducing sugars released was measured using the dinitrosalicylic acid (DNS) method. For the DNS assay, 100 µL of the reaction supernatant was mixed with 100 µL of DNS reagent, incubated at 99°C for 10 minutes, cooled on ice for 5 minutes, and then diluted with 800 µL of double-distilled water. The absorbance was measured at 540 nm, using D-mannose (1–5 µmol/mL) as the standard. One unit (U) of mannanase activity was defined as the amount of enzyme that releases 1 µmol of mannose equivalent per minute under the assay conditions. All assays were performed in triplicate, and the standard deviation did not exceed 5%.

2.5. Stability of ManB-displaying L. plantarum

One milliliter of *L. plantarum* cells displaying surface-bound mannanase, harvested from an overnight culture, was mixed with 0.5 mL of 50% glycerol prior to storage at –20°C. The mannanase activity of the stored cells was assessed at various time points: 1, 2, 3, 4, 5, 6, 7, 60, and 75 days.

2.6. Statistical analysis

All experiments and measurements were carried out at least in triplicate, and the data are given as the mean ± standard deviation when appropriate. The standard deviation was always less than 5%.

3. Results and discussion

3.1. Effect of immobilization on the growth of L. plantarum cells

The growth of recombinant strains carrying constitutive expression vectors for secretion and surface immobilization (pSlpA_1261ManB and pPgm_1261ManB) was evaluated and compared to that of strains carrying the inducible expression vector pSIP_1261ManB (positive control) and the empty vector pEV lacking the *manB* gene (negative control). To assess the effect of enzyme immobilization on host cell growth, optical density was measured at 0 and 4 hours of cultivation. As Figure 1 illustrates, the strain harboring pSlpA_1261ManB exhibited the lowest growth

(OD_{600} ~ 0.65), whereas the negative control strain pEV showed the highest growth (OD_{600} ~ 1.5). Interestingly, the strain carrying the constitutive vector with the Pgm promoter grew better (OD_{600} ~ 0.9) than the strain using the SlpA promoter. As expected, the constitutively expressing strains showed reduced growth compared to the strain with the inducible promoter system (OD_{600} ~ 1.1). These results suggest that the secretion and surface display of ManB imposes a metabolic burden on the host cells, resulting in reduced growth in recombinant strains. A similar observation was reported in another study, where recombinant *L. plantarum* expressing surface-immobilized CCL3Gag protein also showed growth inhibition [11]. However, these findings contrast with reports from Gram-negative surface display systems [12], suggesting that growth responses to surface immobilization may differ clearly between Gram-positive and Gram-negative bacteria.

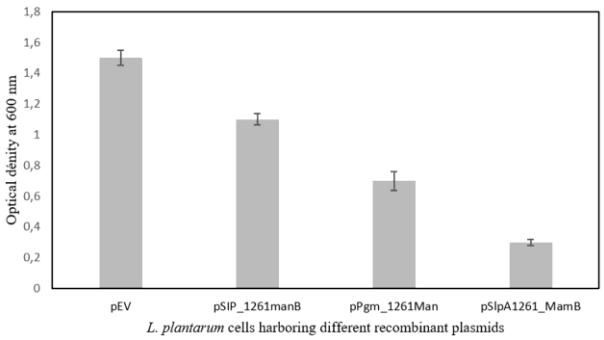

Figure 1. *Growth of recombinant L. plantarum strains. OD_{600} values were measured at the initial time point (0 h; black bars, OD_{600} ~ 0.1) and after 4 hours of cultivation (gray bars) following inoculation. For the strain harboring the inducible promoter (pSIP_1261ManB), recombinant mannanase expression was induced at OD_{600} ~ 0.3 by adding IP-673, and cell growth was measured 2 hours post-induction. Data represent the mean values from three independent experiments*

3.2. Effect of pH control on the growth of recombinant L. plantarum strains

To evaluate the effect of pH on the growth of recombinant strains, cell growth were recorded under both pH-controlled and uncontrolled cultivation conditions. As shown in Figure 2, during the first 2 hours of cultivation, cells exhibited similar growth rates regardless of pH (OD_{600} ~ 0.3–0.4). A significant increase in cell density was observed from 3 h of cultivation with OD_{600} of 1.2 – 1.5 under pH-controlled conditions and 0.65–0.9 without pH control. At 10 h of cultivation, OD_{600} of cultivation with pH control (10.3–10.0) reached to highest growth level and two times higher than the case of cultivation without pH control (~5.3–5.6). Interestingly, after 24 hours, a decline in cell growth was observed in the pH-controlled cultures (OD_{600} ~ 4.7–5.4), potentially due to nutrient depletion. In contrast, an increase in bacterial growth (OD_{600} ~ 8.1–8.2) was observed in the cultures without pH control. It is likely that these bacterial cells had adapted to the acidic conditions and utilized the remaining nutrients more effectively. These results suggest that the optimal time for harvesting large quantities of *L. plantarum* cells displaying surface-bound ManB is at 8 hours of cultivation, which corresponds to the late exponential growth phase.

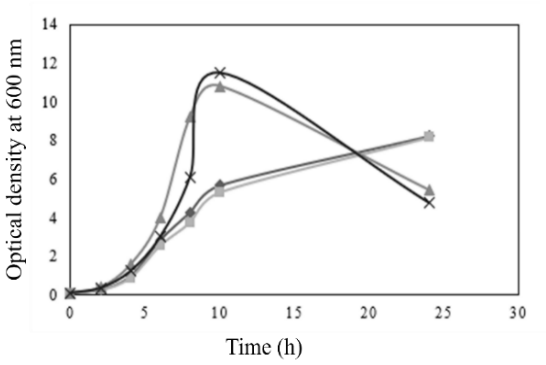

Figure 2. *Growth curves of L. plantarum strains harboring different recombinant plasmids under pH-controlled and uncontrolled cultivation conditions. Cell growth was monitored over time for strains carrying pPgm_1261ManB (gray lines) and pSlpA_1261ManB (black lines) under both pH-controlled conditions [gray line with triangles for pPgm_1261ManB, black line with crosses for pSlpA_1261ManB] and uncontrolled pH conditions [gray line with squares for pPgm_1261ManB, black line with diamonds for pSlpA_1261ManB]*

3.3. Effect of pH control on the catalytic activity of L. plantarum surface-immobilized ManB

During fermentation, *L. plantarum* produces lactic acid, which leads to a decrease in the pH of the culture medium. This pH reduction may negatively affect the catalytic activity of ManB immobilized on the cell surface. Therefore, in this study, *L. plantarum* strains harboring the recombinant plasmids pPgm_1261ManB and pSlpA_1261ManB were cultivated under constant pH conditions (pH=6.0). Mannanase activity was measured at 4, 5, 6, 7, and 8 hours after the innoculation.

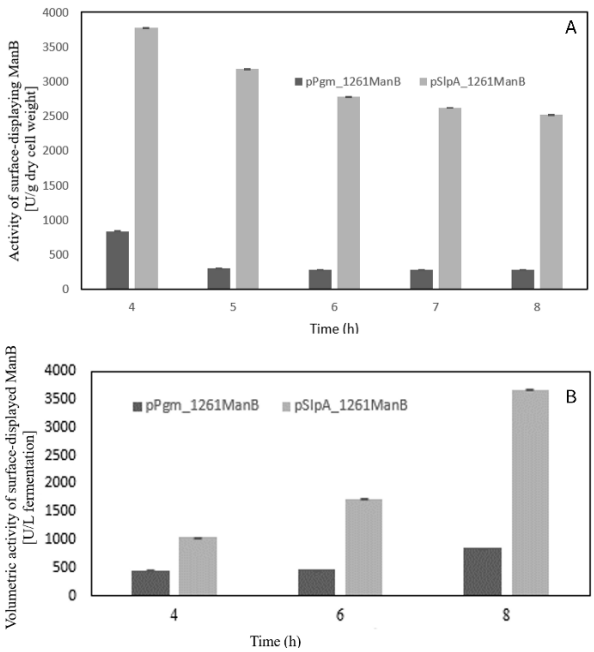

Figure 3. *Enzymatic activity of ManB-displaying cells. Time course of pH-controlled cultivations of ManB-displaying L. plantarum recombinant strains carrying the plasmids pPgm_1261ManB (black bars) and pSlpA_1261ManB (gray bars) in MRS medium with activities of surface displayed mannanase expressed as U/g dry cell weight (A) and U/L fermentation (B). Mannanase activity was determined at each time point*

As shown in Figure 3A, after 4 hours, the strain expressing ManB under the control of the constitutive SlpA promoter exhibited a mannanase activity of approximately 3780 U/g dry cell weight (DCW), which was 4.5 times higher than that of the strain expressing ManB using the Pgm promoter (~842 U/g DCW). At the 5-hour, the activity in the SlpA-driven *L. plantarum* strain decreased to about 3100 U/g DCW, while in the Pgm-driven *L. plantarum* strain, activity dropped by more than half, down to ~308 U/g DCW.

When cultivation was extended to 8 hours, the SlpA strain's activity further declined by about 1.5-fold compared to its 4-hour value, while the activity of the Pgm strain stabilized at approximately 282 U/g DCW from hour 6 to 8. Furthermore, under uncontrolled pH conditions, the activity after 8 hours was remarkably lower: only ~610 U/g DCW for the SlpA strain and ~180 U/g DCW for the Pgm strain [7]. These results indicate that the pH drop during *L. plantarum* growth adversely affects the catalytic efficiency of surface-immobilized ManB. Thus, it is essential to cultivate the recombinant *Lactobacillus* cells in bioreactors equipped with pH control systems to obtain large quantities of *L. plantarum* biomass with high activity of surface-displayed mannanase.

3.4. Effect of pH on the volumetric activity of surface-immobilized ManB in L. plantarum

Under uncontrolled pH conditions, the volumetric activity of ManB immobilized on the surface of *L. plantarum* cells carrying the SlpA and Pgm promoters reached peak values at 6 hours of cultivation [7]. Although cell density continued to increase beyond this point, the volumetric activity of surface-bound ManB declined sharply after 6 hours [7]. This suggests that the decrease of pH may have either denatured the catalytic center of the enzyme or reduced the amount of ManB displayed on the cell surface.

To validate the above hypothesis, the volumetric activity of ManB immobilized on the surface of *Lactobacillus plantarum* cells was assessed under pH-controlled conditions at 4, 6, and 8 hours of cultivation. As shown in Figure 3B, when recombinant cells expressing ManB under the control of constitutive promoters Pgm or SlpA were cultured in a bioreactor with pH maintained constantly at 6.0 using NaOH, a highly increase in volumetric activity was observed from 4 to 8 hours. For the SlpA-driven strain, volumetric activity reached approximately 3670 U/L at 8 hours, which was twice the activity recorded at 6 hours (1700 U/L). Although Pgm-driven strains exhibited lower activity than the SlpA strain, the volumetric activity at 8 hours (850 U/L) was also nearly double that at 6 hours (~462 U/L). This suggest that upholding a constant pH of 6.0 during fermentation not only enhanced activity of ManB displayed on the cell surface but also facilitated *Lactobacillus* cells' growth.

The results of this study also align with previous research by Tien Thanh et al., who found that maintaining pH at a value of 6.5 throughout *L. plantarum* fermentation clearly promoted the growth of recombinant *Lactobacillus* bacteria and enhanced specific and volumetric β-galactosidase activities [13]. Other studies also indicated that pH directly impacts the structure and function of enzymes as well as other proteins that are involved in nutrient uptake or waste efflux within the cells [14-15]. Suboptimal pH can have a negative effect on cell integrity and metabolism, leading to slower growth, reduced biomass, and thus lower overall enzyme production. It is important to know that each enzyme has its own optimal pH range for activity and stability, which may not always be identical to the host's optimal growth pH [15]. Therefore, determining the optimal pH for both the host cell's growth and enzyme production is foundational for high yields.

3.5. Stability of surface-displayed ManB

To evaluate the stability of surface-bound ManB during storage at −20°C, 1 mL of recombinant *L. plantarum* cell suspension expressing ManB under the control of the SlpA promoter was mixed with 0.5 mL of 50% glycerol. The enzymatic activity of ManB immobilized on the cell surface was monitored over time as described in the *Materials and Methods* section. As Figure 4 shows, the activity of surface-immobilized ManB remained stable for the first 2 days under both storage conditions (with or without glycerol). From day 3, a noticeable increase in enzyme activity was observed in the samples stored with glycerol, while activity in the samples stored without glycerol remained unchanged compared to the initial time point. Between days 60 and 75, a slight decline in activity (~92% retention) was recorded in the glycerol-supplemented group, whereas the group stored without glycerol retained only about 70% of the initial activity. These findings indicate that adding glycerol enhances the stability of surface-immobilized ManB during long-term storage at −20°C. Notably, ManB displayed >90% substrate-cleaving efficiency after 75 days of storage when expressed under the constitutive SlpA promoter.

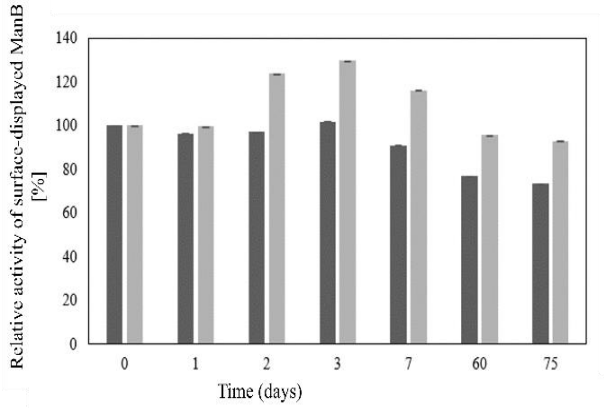

Figure 4. *Relative activity of surface-immobilized ManB on L. plantarum cells under the control of the SlpA promoter during storage at −20°C. Cells were stored either without glycerol (black bars) or with glycerol (gray bars)*

4. Conclusion

We successfully investigated the effect of pH on the activity of continuously surface-immobilized β-mannanase on *Lactobacillus plantarum* cells using the constitutive promoters Pgm and SlpA. The results demonstrated that maintaining pH control during the cultivation of *L. plantarum* expressing surface-bound ManB is essential, as it prevents the denaturation of ManB and enhance the cell growth.

Acknowledgments: HMN would like to thank the Prof. Dietmar Haltrich and Dr. Thu-Ha Nguyen at the University of Natural Resources and Life Sciences (BOKU), Vienna, Austria for their valuable support and guidance during the course of this research.

REFERENCES

[1] E. García-Fruitós, "Lactic Acid Bacteria: a promising alternative for recombinant protein production", *Microbial Cell Factory*, vol. 11, no. 1, p. 157, 2012. https://doi.org/10.1186/1475-2859-11-157

[2] M. Kleerebezem *et al.*, "Complete genome sequence of *Lactobacillus plantarum* WCFS1", *PNAS*, vol. 100, no. 4, pp. 1990–1995, 2003. https://doi.org/10.1073/pnas.0337704100

[3] E. Morello, "Lactococcus lactis, an efficient cell factory for recombinant protein production and secretion", *Journal of Molecular Microbiology and Biotechnology*, vol. 14, no. 1–3, pp. 48–58, 2008. https://doi.org/10.3390/biom12020180

[4] C. Songsiriritthigul, B. Buranabanyat, D. Haltrich, and M. Yamabhai, "Efficient recombinant expression and secretion of a thermostable GH26 mannan licheniformis in *Escherichia coli*", *Microbial Cell Factory*, vol. 9, no. 20, pp. 1–13, 2010. https://doi.org/10.1186/1475-2859-9-20

[5] M. Ibuki, K. Fukui, H. Kanatani, and Y. Mine, "Anti-Inflammatory Effects of Mannanase-Hydrolyzed Copra Meal in a Porcine Model of Colitis", *Journal of Veterinary Medical Science*, vol. 76, no. 5, pp. 645–651, 2014. https://doi.org/10.1292/jvms.13-0424

[6] C. Michon, P. Langella, V. G. H. Eijsink, G. Mathiesen, and J. M. Chatel, "Display of recombinant proteins at the surface of lactic acid bacteria: strategies and applications", *Microbial Cell Factory*, vol. 15, no. 1, pp. 1–16, 2016. https://doi.org/10.1186/s12934-016-0468-9

[7] H.-M. Nguyen *et al.*, "Constitutive expression and cell-surface display of a bacterial β-mannanase in *Lactobacillus plantarum", Microbial Cell Factory,* vol. 18, no. 1, p. 76, 2019. https://doi.org/10.1186/s12934-019-1124-y

[8] X. Yang, Z. Jiang, H. Song, S. Jiang, C. Madzak, and L. Ma, "Cell-surface display of the active mannanase in Yarrowia lipolytica with a novel surface-display system", *Biotechnologial and Applied* Biochemitry, vol. 54, no. 3, pp. 171-176, 2009. https://doi.org/10.1042/ba20090222

[9] L. Fredriksen, C. R. Kleiveland, L. T. O. Hult, C. Nygaard, V. Eijsink, and G. Mathiesen, "Surface display of N-terminally anchored invasin by *Lactobacillus plantarum* activates NF-κB in monocytes", *Applied and Environmental Microbiology*, vol. 78, no. 16, pp. 5864–71, 2012. https://doi.org/10.1128/AEM.01227-12

[10] H. M. Nguyen *et al.*, "Display of a β - mannanase and a chitosanase on the cell surface of Lactobacillus plantarum towards the development of whole - cell biocatalysts", *Microbial Cell Factory*, vol. 15, no. 1, pp. 1–14, 2016. https://doi.org/10.1186/s12934-016-0570-z

[11] K. Kuczkowska, G. Mathiesen, V. G. H. Eijsink, and I. Øynebråten, "Lactobacillus plantarum displaying CCL3 chemokine in fusion with HIV-1 Gag derived antigen causes increased recruitment of T cells", *Microbial Cell Factory*, vol. 14, no. 1, p. 169, 2015. https://doi.org/10.1186/s12934-015-0360-z

[12] I. E. P. Tozakidis, S. Sichwart, and J. Jose, "Going beyond *E. coli*: autotransporter based surface display on alternative host organisms", *New Biotechnology*, vol. 32, no. 6, pp. 644–650, 2015. https://doi.org/10.1016/j.nbt.2014.12.008

[13] T. T. Nguyen *et al.*, "Heterologous expression of a recombinant lactobacillal β-galactosidase in Lactobacillus plantarum: Effect of different parameters on the sakacin P-based expression system", *Microb. Cell Fact.*, vol. 14, no. 1, pp. 1–12, 2015. https://doi.org/10.1186/s12934-015-0214-8

[14] E. A. K. Talley, "On the pH-optimum of activity and stability of proteins", *Proteins*, vol. 78, no. 12, pp. 2699–2706, 2008. https://doi.org/10.1002/prot.22786

[15] D. T. Edward P, O Brien, and B. R. Brooks, "Effects of pH on proteins: Predictions for ensemble and single molecule pulling experiments", *J. Am. Chem. Soc.*, vol. 134, no. 2, pp. 301–314, 2013. https://doi.org/10.1021/ja206557y