Egyptian Journal of Chemistry
http://ejchem.journals.ekb.eg/

Statistical Optimization of β-galactosidase Production from Newly Isolated *Bacillus licheniformis* 17KAN-M3 Strain

Sara A. Kassem[1], Shireen A.A. Saleh[1], Mohamed A. Abdel-Naby[1], Naziha M. Hassanein[2], Samia A. Ahmed [1,*]

[1] *Chemistry of Natural and Microbial Products Department, National Research Centre, Dokki, Giza, Egypt*
[2] *Microbiology Department, Faculty of Science, Ain Shams University, Abbaseyya, 1156 Cairo, Egypt*

Abstract

β-galactosidase (EC 3.2.1.23) is the key enzyme in the dairy sector regarding the production of low-lactose foods to treat lactose intolerance. The present study aimed to identify the most effective bacterial isolates from different dairy product samples and screened for β-galactosidase (β gal) production. Among thirteen bacterial isolates, the highest β-gal production (2245 U/ml) was obtained by isolate no.6 which was isolated from fresh cheese. The potent isolate was identified and submitted to the gene bank as *Bacillus licheniformis* strain 17KAN-M3 (accession number OR742338). Some fermentation parameters were optimized separately via one–variable-at-a-time (OVAT) including incubation time, carbon sources, incubation temperature, initial pH, and inoculation level. Statistical factorial designs including a series of statistical designs, such as the Plackett–Burman design (PBd) and Central Composite design (CCd) was used to optimize the fermentation medium composition. Using the revised medium, the productivity of β-gal from *Bacillus licheniformis* 17KAN-M3 (14048 U/ml) was 6.26-fold higher than that produced from the basal medium. Since its ability to produce β-gal was greatly improved by RSM, *Bacillus licheniformis* strain 17KAN-M3 could serve as a potential source of β-gal for industrial-scale food applications.

Keywords: β-galactosidase; optimization; response surface methodology; milk whey.

1. Introduction

Enzymes play a very important role in many industries. β-galactosidase (β-gal) also known as lactase, an enzyme capable of hydrolyzing lactose into glucose and galactose, is of great interest due to its wide range of applications **[1]**. It hydrolyzes lactose, which has been widely exploited in food industries, as it works to relieve lactose indigestion, prevent lactose crystallization, and enhance the flavor, sweetness, and solubility of lactose **[2-4].** On the other hand, lactose hydrolysis helps reduce lactose levels in whey (by-products) resulting from cheese manufacturing processes **[5].** GOS represents an important functional food component, as it is a vital substance (prebiotic) for gut health **[5]**. The special application β -gal enzyme for the production of galactooligosaccharides (GOS) by acting on lactose through transgalactosylation (which is the component of prebiotics) has been reported **[6]**.About 60% of the world's population has difficulty in digesting lactose, leading to a syndrome called lactose intolerance, which reduces their quality of life **[7]**.

Lactose can be hydrolyzed by acid and enzymatic methods. The process of acid hydrolysis of lactose is fast. However, this process requires high temperature which causes a change in the color and smell of the product. This is negatively affecting its use in the food industry **[4]**. On the other hand, the enzymatic hydrolysis can be applied safely in dairy industries without pretreatment and purification. Further, the properties of the resulting products related to the raw materials are preserved **[4]**. The demand for dairy products rich in β-gal is increasing day by day, especially because it hydrolyses lactose without affecting the taste or nutritional value **[8]**.

β-gal can be found in plant, animal, and microbial sources. However, most of industrial application uses β-gal from microbial sources due to their high reproduction rate, high yield of enzyme production, greater catalytic activity, stability, and ease of handling **[2-9]**.

The cost of production of biocatalyst is the key parameter controlling its uses in industrial applications. We can overcome this problem by using hyper-producers microbial strains, finding the optimum conditions for their production, and using a low-cost substrate (by-product) **[10]**. Furthermore, the resulting by product produced from enzymatic bioprocesses can be converted into high-value-added products. Consequently, this will reduce the cost of the process and lower the pollution load **[11]**.

Cheese factories around the world produce more than 121 × 10^6 tons of whey annually. Given the environmental risks posed by whey and the amount of lactose it contains, ways must be found to reuse whey [4]. In the dairy industry, whey is the main by-product that causes environmental pollution due to its high production

*Corresponding author e-mail: dr_sa_ahmed@yahool.com.; (Samia Abdel-Aziz Ahmed).
Received date 09 October 2024; Revised date 24 December 2024; Accepted date 05 January 2025
DOI: 10.21608/EJCHEM.2025.327019.10603

volumes and organic load. About half of the whey production is disposed of in wastewater treatment plants or reused on farms. Whey has an interesting composition shows great potential for use in bioprocessing to obtain bioactive compounds such as β-gal enzyme with low cost **[10]**.

Whey is the main byproduct of dairy industry. Its volumetric contribution of milk is 85 – 90 %. The major components of whey are lactose (4.5 – 5%), amino acids and proteins (6 – 8%), and minerals 4-9% **[4]**. With its high nutritional value and amazing ability to be contaminated, the reuse of whey has attracted worldwide attention **[5]**.

The production of microbial enzymes including β-gal is greatly influenced primarily by the type of microbial strain used, the physical parameters of the fermentation, and the composition of the production medium. Optimizing enzyme production using only one-variable-at-a-time (OVAT) method is practically ineffective since it takes long time, and often impractical. This is partially so when large number of variables need to be studied **[11]**. Response Surface Methodology (RSM) is a statistical model used to enhance the efficiency of production processes. Furthermore, RSM reduces the number of individual experiments needed, which enhances the information obtained as well as the knowledge of the interactions between different variables.

The present work aims to isolate and identify bacterial strain efficient in producing β-galactosidase (β-gal) enzyme. Improve the yield of enzyme production initially by using the one–variable-at-a-time (OVAT) method. Finally, optimize the significant variables using multi-factorial designs (Plackett–Burman PB followed by Central Composite CC) and the model is verified.

2. Materials and Methods

2.1. Materials

O-Nitrophenyl-beta-D-galactopyranosidase (ONPG) was obtained from Sigma-Aldrich (St.Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Isolation of microorganism

Different dairy product samples such as fresh cheese, spoiled cheese, fresh yoghurt, spoiled yoghurt, fresh milk, and spoiled milk were used as a source of isolates. The samples from liquid sources were taken directly. The solid sources (10 g) were suspended in 100 ml of sterile H_2O for 30 min at 35 °C and 200 rpm on a rotator shaker. 0.1 ml of the suspension was spread with a needle on nutrient agar (NA) plates and incubated at 30 °C. To purify isolate, the bacterial colony was repeatedly transferred to NA slants until the colony was deemed uniform. Purified isolate was maintained at 4 °C on NA slants. The purified isolate was screened for β-gal production.

2.3. Inoculum preparation

The bacterial suspension was prepared by scratching 2 days old NA slants for bacteria with 5 ml sterile H_2O using a sterile needle. This suspension was used to inoculate each fermentation flask (250-ml Erlenmeyer flask) **[12]**.

2.4.β-gal production under submerged fermentation conditions

The basic medium for enzyme production was composed of (g/l): Lactose 10, beef extract 15, peptone 5, yeast extract 0.5, and sodium chloride 1.5 at initial pH 6 **[13]**. After inoculation with 5 ml of inoculum per flask containing 50 ml production medium, the flasks were incubated at 35 °C for 48 h and 150 rpm.

2.5. β-gal assay

The β-gal activity was carried out according to Hsu et al. **[14].** The activity was expressed as µM of O-Nitrophenyl released per min. The reaction mixture in a final volume of one ml was composed of 0.5 ml of culture filtrate (or enzyme solution) and 0.5 ml of ONPG as substrate dissolved in 15 mM in phosphate buffer (pH 6.5). The reaction mixture was incubated at 37 °C for 20 min. The catalytic reaction was stopped by the addition of two ml of Na_2CO_3 solution (0.1 M). The absorbance of the developed color was read at 420 nm. One unit of β-gal activity was expressed as the amount of enzyme that releases one µM of o-nitrophenol per min.

2.6. Nucleotide sequence and molecular identification

This was done for the potent bacterial isolate was at the Nawah center (HQ: 8 Jan van Goyenkade, 1075 HP Amsterdam, Netherlands. Labs: Al-Mokattam mall, Cairo, Egypt), ww.nawah-scientific.com. Then, the nucleotide sequence was deposited at the NCBI database as Bacillus licheniformis strain 17KAN-M3 (GenBank accession number OR742338).

2.7. Optimization of β-gal production by one- variable -at-a-time (OVAT)

2.7.1. Effect of incubation period

β-gal production was performed by growing bacterial cells in the basal medium as described above at different incubation periods (24-120 h). The clear culture filtrate was investigated for of β-gal activity **[15]**.

2.7.2. Effect of carbon source

Lactose from the basal medium (at 1.0% w/v) was replaced with glucose, maltose, sucrose, galactose, and dextrin. The fermentation was conducted incubated at 35 °C for 72 h with agitation speed of 150 rpm. At the end of fermentation the culture filtrate was assayed for β-gal **[4]**.

2.7.3. Effect of fermentation temperature

Optimum temperature for β-gal enzyme production was checked in the range of 30–45 °C. The fermentation was conducted for 72 h and 150 rpm **[12]**.

2.7.4. Effect of initial pH

The production of β-gal was carried out by growing the bacterial cells at different pH values ranged from 3 to 8 **[7]**.

2.7.5. Effect of inoculum size

Different volumes of inoculum (2-7 ml) were added per flask. The flasks containing the fermentation media were incubated at 37 °C for 72 h with an agitation speed of 150 rpm and the β-gal activity was determined **[15]**.

2.8. Multi-factorial designs for optimization of β-gal production

The optimization protocol was performed in three steps: (1) evaluation of the important nutrients and conditions for β-gal production according to Plackett-Burman design **[16]**, (2) selection of the most important factors for further estimation of their optimal levels using Central Composite design **[17],** and (3) application of computational analysis to check the goodness of fit of the model as expressed by the coefficient of determination R^2.

2.8.1. First design Plackett-Burman (PBd)

The study examined the effect of 11 variables on β-gal production containing: peptone, yeast extract, NaCl, $MgCl_2$, lactose, glucose, $(NH_4)_2SO_4$, beef extract, $CaCl_2$, milk whey, and tween-80 was investigated. Each of these factors was represented at two levels, low value (-1) and high value (+1) as shown in **Table 1**. Also, in the experimental design, each row represented an experiment, and each column represented an independent variable. The F-value was used to establish statistical significance, and R^2 was used to calculate the proportion of variance described by the model obtained (**Table 2**). Based on regression analysis, the variables that proven a significant effect on β-gal production were estimated in the second optimization design.

2.8.2. Second design Central composite (CCd)

Central Composite design (CCd) was used to optimize the level of variables for enhancing β-gal production. For β-gal production, the three significant variables elucidated through PBd were: milk whey (A), Tween 80 (B), and $(NH_4)_2SO_4$ (C). Each factor was presented at five levels: (-1.682), (-1), (0), (+1), and (+ 1.682) (**Table 3**). Total number of experiments was 20 runs including six central points in a quadratic design model. The ANOVA was performed to prove the significance of design for β-gal activity presented in **Table 4**.

3. Results and discussion

3.1. Selection and identification of the potent isolate for β-gal production

Different dairy product samples (fresh cheese, spoiled cheese, fresh yoghurt, spoiled yoghurt, fresh milk and spoiled milk) were used as a source of isolates that have the ability to produce the β-gal enzyme. The current study was carried out to investigate the efficiency of β-gal production from different bacterial isolates. Thus, thirteen bacterial isolates were obtained from the isolation step and screened for β-gal production to select the most potent producer (data not shown). The maximum β-gal production (2245 U/ml) was noted by isolate no. 6 isolated from fresh cheese. Bacterial β-gal enzymes have been widely used for lactose hydrolysis due to their high enzyme activity, good stability, and high production yield **[4].** The sequence of the potent isolate has been submitted to NCBI and the gene bank was obtained as *Bacillus licheniformis* strain 17KAN-M3 (accession number OR742338). The phylogenetic tree of *B. licheniformis* 17KAN-M3 with the nearest phylogenetic neighbors in NCBI was shown in **Fig. 1**.

The optimization protocol was performed in two steps: one- variable -at-a-time (OVAT) and multi-factorial experiments (RSM).

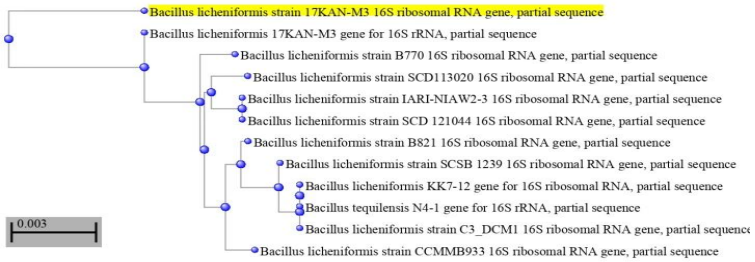

Figure 1: Phylogenetic tree of *B. licheniformis* strain 17KAN-M3 (OR742338) with some closest phylogenetic relatives in NCBI-GenBank.

3.2. Optimization of β-gal enzyme production by one- variable -at-a-time (OVAT)

3.2.1. Effect of incubation period

Incubation time plays an important role in β-gal production. Maximum production of β-gal was observed at 72 h as illustrated in **Fig. 2**. At 96 and 120 h, β-gal production decreased by 1.2 and 1.74-fold, respectively. These results may be due to the consumption of most of the substrates necessary for growth, and / or the production of organic acids that lead to lowering the pH of the medium to levels that prevent bacterial growth **[15]**. **Akcan [16]** noted that maximum amount of β-gal production by *B. licheniformis* ATCC 12759 was obtained at 120 h. On the other hand, maximal β-gal production by *Lactobacillus leichmannii* was achieved after 12 h of growth **[15].**

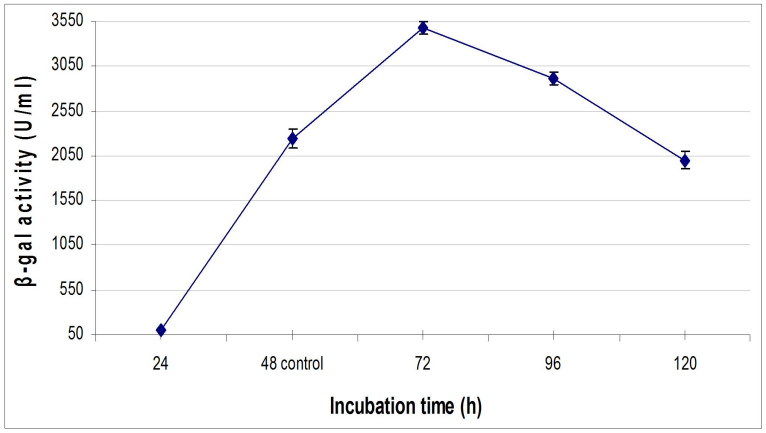

Figure 2: Effect of different incubation period on β-gal production by *B. licheniformis* strain 17KAN-M3.

3.2.2. Effect of carbon source

Several studies have documented how carbon sources significantly affect the synthesis of β-gal by different bacteria. In the present study lactose from the basal media was replaced by different carbon sources (1.0% w/v, glucose, maltose, sucrose, galactose, and dextrin) and they investigated for β-gal production. As shown in **Fig. 3**, the most effective carbon source to obtain maximum β-gal production (3241 U/ml) was found to be lactose. This result was 2.77- fold higher than that reported by **Anbalagan and Vaithilingam [7]**. In addition, β-gal production was significantly suppressed when *B. licheniformis* strain 17KAN-M3 was grown on a glucose-containing medium. Similarly, **Akcan [16]** described that addition of glucose to the growth medium inhibit the synthesis of β-gal by bacterial strain. Glucose inhibits the production of β-gal through metabolic suppressive mechanisms **[15]. Abdaltef et al. [1]** reported that lactose considerably increased β-gal production by *Lactobacillus* spp. A similar result was obtained by **Vasudha and Gayathri [4]** when lactose significantly increased the production of β- gal by *Lactiplantibacillus plantarum* GV54 isolate.

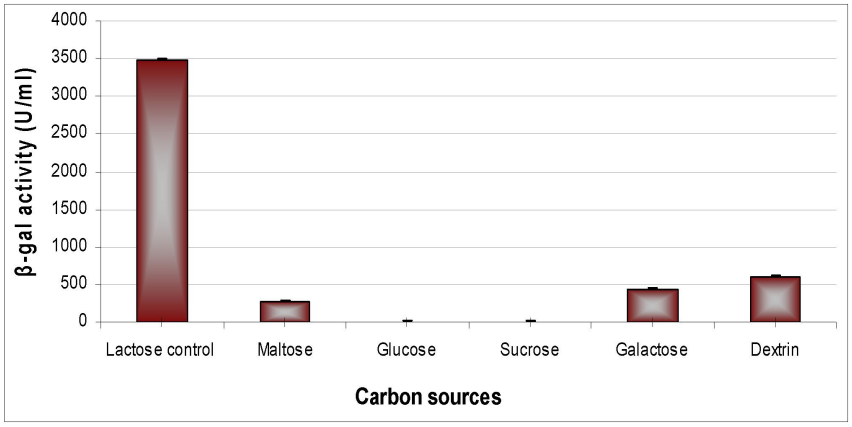

Figure 3: Effect of different carbon sources on β-gal production by *B. licheniformis* strain 17KAN-M3.

3.2.3. Effect of fermentation temperature

In the present investigation the effect of fermentation temperature on β-gal production showed that the enzyme yield increased by 1.22-fold as the incubation temperature increased from 35 to 37 °C (**Fig. 4**). This optimum temperature for β-gal production was similar to the results reported by **Anbalagan, and Vaithilingam [7]**. Highest β-gal production using *Kluyveromyces marxianus* CCT 4086 was reached at 30°C **[12].**

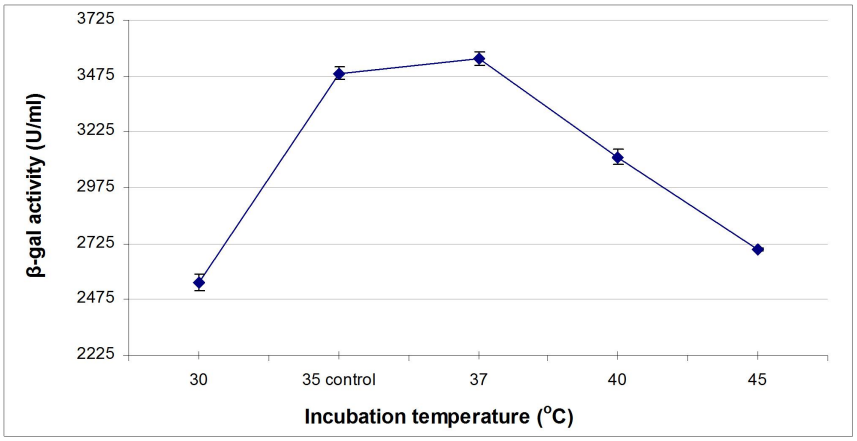

Figure 4: Effect of different fermentation temperature on β-gal production by *B. licheniformis* strain 17KAN-M3.

Higher fermentation temperatures (more than 37°C), had an adverse effect on β-gal yield. This may be due to the negative effect of higher temperature (more than 37°C) on the metabolic bath way of enzyme biosynthesis. In addition, higher incubation temperature (more than 37°C), prevents enzyme formation, possibly by inhibiting cell viability and enzyme stability **[18]**. In contrast, low temperature values may slow down the metabolism of the microorganism and consequently, enzyme production **[10]**.

3.2.4. Effect of initial pH

The effect of initial pH of the fermentation medium (from pH 3.0 – 8.0) on enzyme production was investigated. Results showed that maximum β-gal production by *B. licheniformis* 17KAN-M3 was observed at pH 6 (**Fig. 5**) and it decreased above and below the optimum pH. **Abdaltef et al. [1]** reported that maximum β-gal production by *Lactobacillus* spp at pH 7.0. Also, the highest β-gal production by *Kluyveromyces marxianus* and *Lactobacillus plantarum* was reached in pH 7.0 **[7-12].**

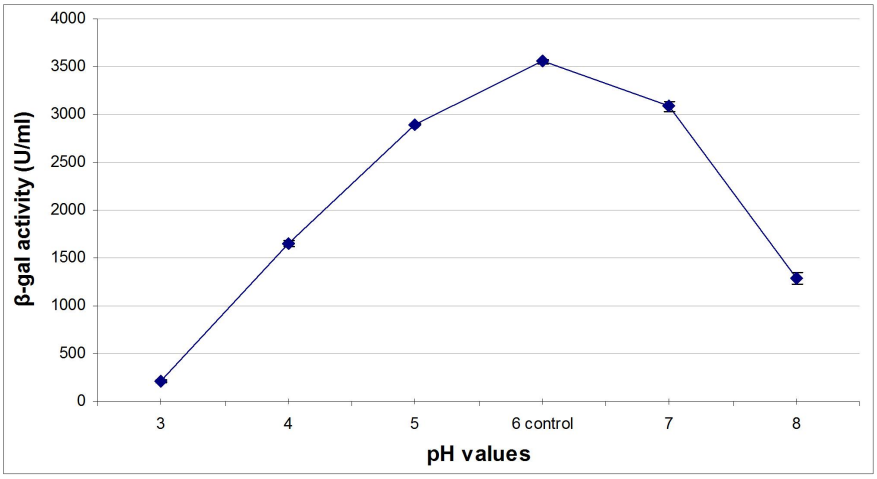

Figure 5: Effect of initial pH on β-gal production by *B. licheniformis* strain 17KAN-M3.

3.2.5. Effect of inoculum size on β-gal production

The highest β-gal production was obtained at an inoculum level of 10% (v/v) (**Fig. 6**). Using larger inoculum size (more than 10% v/v) led to gradual decreases of growth and enzyme biosynthesis. A lower inoculum size may require a longer time for fermentation and enzyme production **[15].** On the other hand, larger inoculum level (35%) was reported for maximum β-gal production by *B. licheniformis* ATCC 12759 **[15].** The result obtained from OVAT showed 3555 U/ml of β-gal activity which was 1.60- fold higher as compared to un-optimized conditions. **Anbalagan and Vaithilingam [7]** reported that the maximum experimental value based on OVAT for β-gal production was lower than our result by 3.1-fold.

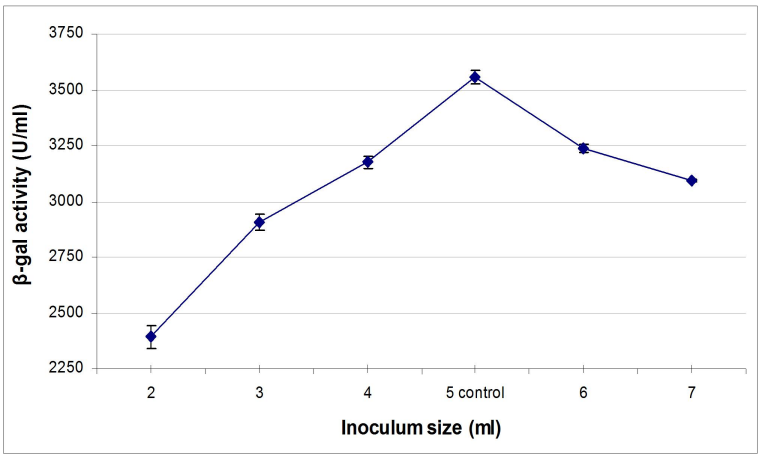

Figure 6: Effect of inoculum size on β-gal production by *B. licheniformis* strain 17KAN-M3.

3.3. Multi-factorial designs for optimization of β-gal production

3.3.1. First design Plackett-Burman (PBd)

Statistical factorial design optimization helps save time and effort, compared to the traditional optimization method by OVAT, because it combines statistical and mathematical techniques to design experiments, build models, and explore the relationships between many independent variables in the system response **[6].** PBd is an efficient technique for optimization, which was first used to pick variables that significantly influenced enzymes production.

As shown in **Table 1**, PBd was used to detect the relative importance of different medium components (11 variables) on β-gal production. The highest β-gal production (6278 U/ml) was obtained in trial 10. Out of the 11 variables tested, milk whey, tween 80, and ammonium sulfate had positive impact on β-gal production while glucose, lactose, $CaCl_2$, and peptone had a negative impact. On the other hand, yeast extract, NaCl, $MgCl_2$, and beef extract have no significant effect on β-gal enzyme production as shown in Pareto chart (**Fig. 7A**).

Numerous studies had documented how carbon and nitrogen sources have a significant impact on the β-gal production by different bacteria. As mentioned in **Fig. 7A**, ammonium sulphate was the best nitrogen source for β-gal production. On the other hand, yeast extract was the best nitrogen sources for β-gal production by *Paracoccus marcusii* **[19].** Similar observation was recorded by **Alikunju et al. [20]** as noted via PBd that whey had a positive effect on β-gal production by *Enterobacter ludwigii*. Rice straw and orange peel wastes were used as cheap and eco-friendly substrates in the production of β-galactosidase by *L. paracasei* MK852178 **[8]**.

Table 1: Plackett-Burman design for *Bacillus Licheniformis* 17KAN-M3 β-gal production

Run	Variable code											β-gal activity (U/ml)
	1: A	2: B	3: C	4: D	5: E	6: F	7: G	8: H	9: J	10: K	11: L	
	Peptone (g/l)	Yeast Extract (g/l)	NaCl (g/l)	$MgCl_2$ (g/l)	Lactose (g/l)	Glucose (g/l)	$(NH_4)_2SO_4$ (g/l)	Beef extract (g/l)	$CaCl_2$ (g/l)	Milk whey (ml/l)	Tween 80 (ml/l)	
1	1	2	0	2	10	0	2	15	2	0	0	469
2	5	0.5	1.5	2	2	5	2	15	0	0	0	5
3	5	0.5	0	0	10	0	2	15	0	10	1	5415
4	1	0.5	0	2	2	5	2	5	2	10	1	4716
5	1	0.5	1.5	0	10	5	0	15	2	10	0	85
6	1	2	1.5	0	10	5	2	5	0	0	1	4
7	5	2	1.5	0	2	0	2	5	2	10	0	4238
8	5	2	0	0	2	5	0	15	2	0	1	254
9	1	0.5	0	0	2	0	0	5	0	0	0	1544
10	1	2	1.5	2	2	0	0	15	0	10	1	6278
11	5	2	0	2	10	5	0	5	0	10	0	4
12	5	0.5	1.5	2	10	0	0	5	2	0	1	807

*Row represents an experiment and column represents an independent variable

Whey is the liquid portion of milk remaining after casein has been precipitated and removed. It is one of the most important by-products of the cheese industry, with annual production exceeding 160 million tons worldwide, and an estimated growth rate of 1-2% per year **[6]**. Whey is a valuable waste product that retains about 55% of the nutrients found in milk, including (%) of lactose (4.5–5), proteins (0.6–0.8), lipids (0.4–0.5), mineral salts (8–10 of the dry extract), vitamins, citric acid (0.1), and lactic acid (0.05) thus shows great potential for use in bioprocessing to obtain β-gal enzyme **[12]**.

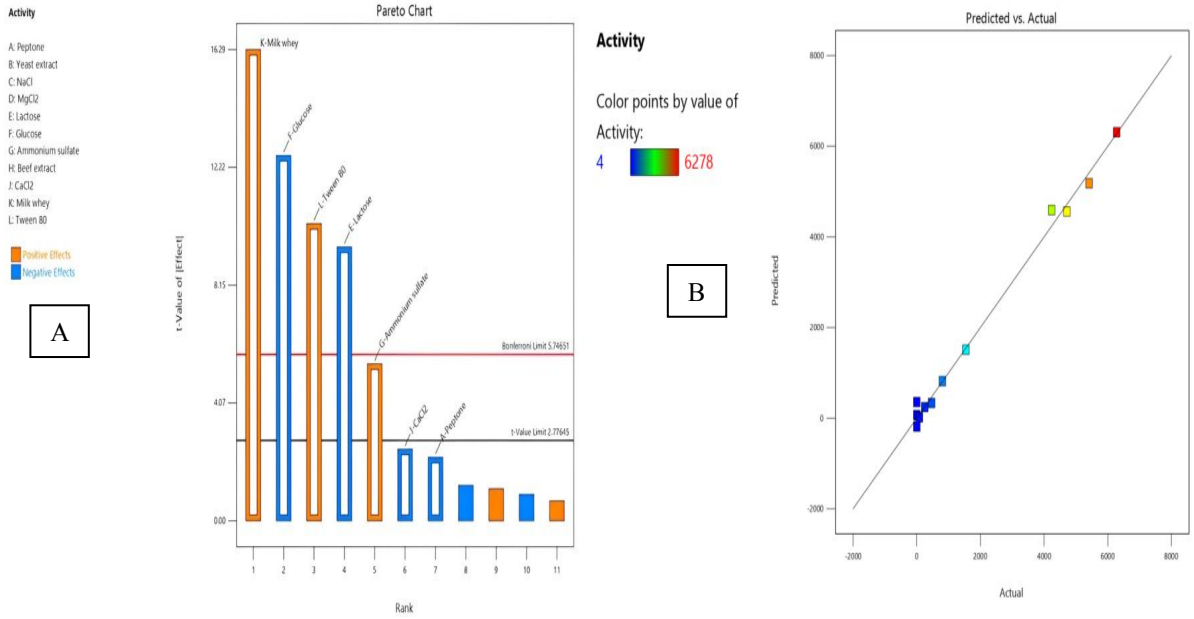

Figure 7: (A) Pareto chart showing the effect of each variable on *B. Licheniformis* 17KAN-M3 β-gal production and (B) Parity plot to show the distribution of observed and predicted values for *B. Licheniformis* 17KAN-M3 β-gal production.

Duan et al. [6] developed a cost-effective whey based medium for culturing *Lactobacillus bulgaricus* to produce β-gal. Six variables, including whey powder, $CH_3COONa.3H_2O$, triammonium citrate, K_2HPO_4, yeast powder, and corn steep liquor, were evaluated via PBd which showed that whey powder had the most positive effect. **Amin et al [21]** reported that peptone, $MgSO_4$, and glucose have the greatest positive effect on enzyme production through PBd. These results are inconsistent to our findings, which confirmed that glucose had a negative effect on enzyme production. However, the positive effect of peptone is against our results. Different result was recorded by **Vasudha and Gayathri [22]** who produced maximum β- gal enzyme using mixture of ammonium sulfate and beef extract as the nitrogen source by *Lactiplantibacillus plantarum* GV54 isolate. Our results go different form **Deng et al. [15]** who reported lactose and pH as positive factors for β- gal enzyme production.

According to the ANOVA results the model was considered to be significant because the F-value of the model was 94.33 and there is only 0.0283% probability that the model is caused by noise. Therefore, milk whey, tween 80, and ammonium sulfate were considered to be significant factors because their "Prob > F" values are lower than 0.05 (**Table 2**).

Table 2: Analysis of variance (ANOVA) for Plackett-Burman design for β-gal production by *B. licheniformis* 17KAN-M3

Source	Sum of Squares	Degree of Freedom (df)	Mean Square	F Value	p-value Prob > F	
Model	64593755	7	9227679	94.33607	0.000283	Significant
A-Peptone	469260.8	1	469260.8	4.797329	0.093663	
E-Lactose	8756917	1	8756917	89.52339	0.000696	
F-Glucose	15602041	1	15602041	159.5022	0.000226	
G-$(NH_4)_2SO_4$	2876302	1	2876302	29.40491	0.005607	
J-$CaCl_2$	598980.1	1	598980.1	6.123471	0.0686	
K-Milk whey	25969034	1	25969034	265.4857	$8.3*10^{-5}$	
L-Tween 80	10321220	1	10321220	105.5155	0.000506	
Residual	391268.3	4	97817.08			
Cor Total	6498523	1				

R^2= 0.9939, adjusted R^2= 0.9834, predicted R^2= 0.9458, Adequate precision= 25.42, Standard deviation= 312.75, Mean= 1984.9, coefficient of variance %= 15.75.

The strength of the model was affirmed by the closeness of the determination coefficient of the model (R^2= 0.9939) to 1. Also, the rational agreement between the predicted R^2 (0.9458) and the adjusted R^2 (0.9834) revealed the good correlation between the observed and predicted activities. For adequate signal of the model, the ratio between the signal to the noise (S/N) should be greater than 4. In the present design the (S/N) was 25.42. The following equation describes the relation between the eleven factors and β-gal production is:

β-gal activity (U/ml) =1984.92 -197.75*Peptone -854.25* Lactose -1140.25*Glucose +489.58* $(NH_4)_2SO_4$-223.42*$CaCl_2$ +1471.08* Milk whey +972.42*Tween 80

The accuracy and validation of the design was confirmed by the closeness between the actual β-gal productivity values and the activity values predicted by the model (**Fig. 7B**). The PBd optimization process increased enzyme production from 3555 U/ml (the OVAT production medium) to 6278 U/ml (trial 10), leading to a 1.77-fold increase in β-gal enzyme production.

3.3.2. Second design Central composite (CCd)

The PBd was effective in detecting the significant medium constituents while CCd identified their optimum concentrations. The CCd (**Table 3**) revealed the optimum concentrations for the three significant factors determined by PBd. According to the ANOVA results the model proved its significance since the F-value of the model was high (178.68). Also, rational agreement was detected between the predicted R^2 (0.95081) and the adjusted R^2 (0.988258) which advocated the fitness and significance of the model (**Table 4**). The closeness of R^2 (0.99382) to 1 proved that the model was robust and predicted enzyme production more accurately. The adequate precision (signal to noise ratio) was 54.6 (more than 4) which indicated an adequate signal.

Table 3: Central composite design for *B. Licheniformis* 17KAN-M3 β-gal production.

Run	Factor 1 A: milk whey [ml/l]	Factor 2 B: Tween 80 [ml/l]	Factor 3 C: $(NH_4)_2SO_4$ [g/l]	β- gala activity [U/ml]
1	10	18.4089	3.5	4409
2	5	5	5	8362
3	15	5	5	5578
4	10	10	6.0227	8371
5	5	5	2	10208
6	5	15	2	6826
7	10	10	3.5	5345
8	15	5	2	6456
9	1.5910	10	3.5	4891
10	10	10	3.5	5345
11	18.4089	10	3.5	6763
12	10	10	3.5	5345
13	15	15	2	11807
14	10	10	3.5	5345
15	15	15	5	7551
16	10	1.5910	3.5	4455
17	10	10	3.5	5345
18	10	10	3.5	5345
19	10	10	0.9773	14048
20	5	15	5	2874

Table 4: Analysis of variance (ANOVA) for CCd design for β-gal production by *B. Licheniformis* 17KAN-M3

Source Model	Sum of Squares	Degree Of Freedom (df)	Mean Square	F Value	p-value Prob > F	
	$1.38*10^8$	9	15370069	178.6838	$7.57*10^{-10}$	Significant
A-milk whey	2878911	1	2878911	33.4686	0.000176	
B-Tween 80	192965.8	1	192965.8	2.243312	0.165077	
C- $NH_4)_2SO_4$	30710694	1	30710694	357.0253	$3.74*10^{-9}$	
AB	32780705	1	32780705	381.0901	$2.72*10^{-9}$	
AC	55112	1	55112	0.640701	0.442058	
BC	3759282	1	3759282	43.7033	$6*10^{-5}$	

A^2	640813.3	1	640813.3	7.449736	0.021215	
B^2	1148786	1	1148786	13.35514	0.004429	
C^2	64396134	1	64396134	748.6333	$9.85*10^{-11}$	
Residual	860182	10	86018.26			
Lack of Fit	860182.6	5	172036.5			
Pure Error	0	5	0			
Cor Total	$1.39*10^{8}$	19				

R^2= 0.993, adjusted R^2= 0. 0.988, predicted R^2= 0.950, Adequate precision= 54.62, Standard deviation= 293.28, Mean= 6733.45, coefficient of variance %= 4.35.

The following equation can used to calculate the β-gal enzyme activity:

β-gal activity (U/ml) = 5338.81 + 459.133*Milk whey -118.868*Tween 80- 1499.58*$(NH_4)_2SO_4$+ 2024.25* Milk whey*Tween 80 + 83*Milk whey*$(NH_4)_2SO_4$ – 685.5*Tween 80*$(NH_4)_2SO_4$

Both normal plot of residuals and perturbation plots indicated the effectiveness and validity of the model. The 3D plot (**Fig. 8**) is the graphical presentations of the main effect and the interactions among the variables that affected significantly β-gal production.

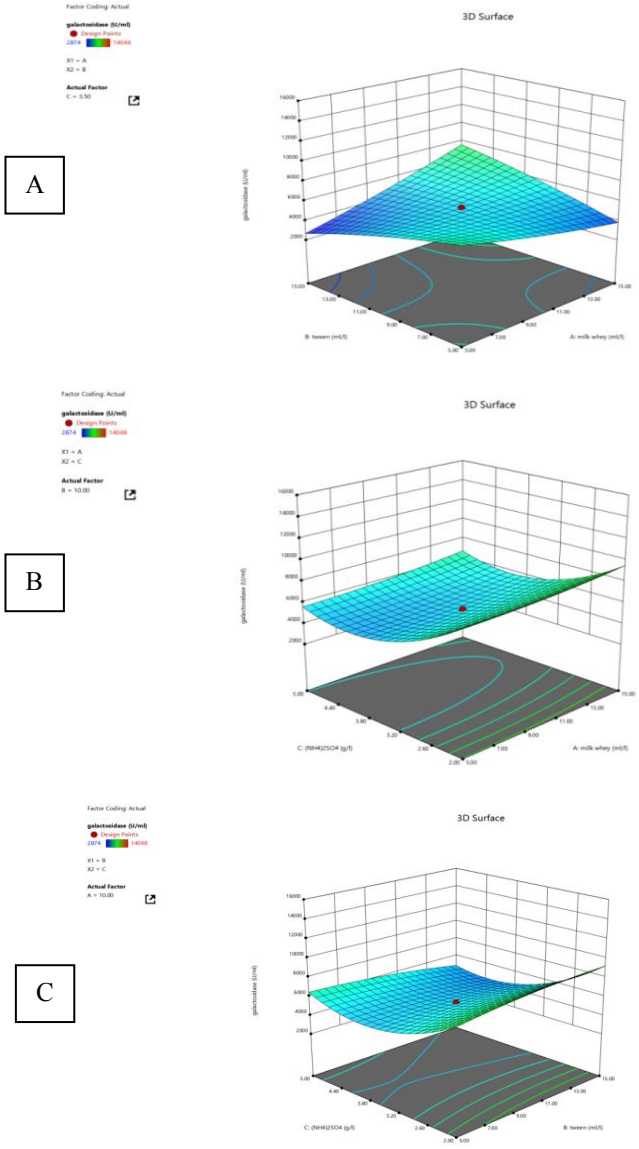

Figure 8: 3D graphs (A,B, C) showing the interaction between significant variables milk whey, $(NH_4)_2SO_4$, and Tween 80.

Validation of the CCd model was confirmed by the reproducibility of results and by the proximity among the actual and predicted β-gal activities (**Fig. 9**). The CCd optimization led to increase β-gal production from 6278 U/ml under the PBd to 14048 U/ml (trial 19), leading to a 2.24-fold increase in β-gal production.

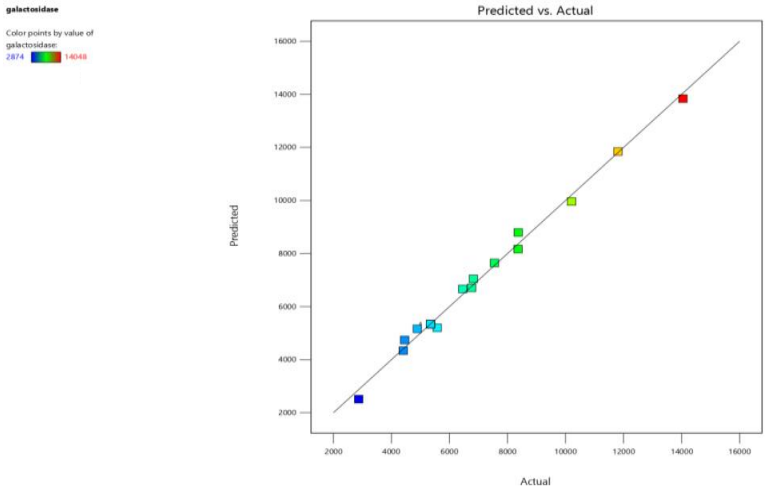

Figure 9: Validation of CCd for optimization of β-gal enzyme production.

Finally, the optimization steps led to a massive increase in β-gal productivity from 2245 U/ml under the basic production medium conditions to 14048 U/ml under optimized conditions. In other words, an overall 6.26-fold increase in β-gal production was achieved after applying the optimization methods. Lower β-gal production (2848U/ml) based on RSM was reported by **Anbalagan and Vaithiyalingam [7]**. In an optimized medium by RSM, 34.37 U/ml of β-gal was produced from *Enterobacter ludwigii* giving a 3.6-fold increase as compared to unoptimized medium **[20]**.

The optimum conditions for β-gal production were achieved through using in 50ml statistically optimized medium inoculated with 5ml of inoculum, (pH 6) for 72 h at 150 rpm in a temperature controlled 37°C. The statistically optimized medium was composed of (g/l): yeast extract, 2; peptone, 1; beef extract, 15; NaCl, 1.5; MgCl, 2; $(NH4)_2SO_4$, 0.98; Lactose, 2 and 10 ml/l of milk whey and Tween 80.

4. Conclusions

Bacterial isolates from dairy product samples were screened for β-gal enzyme production. The potent isolate isolated from fresh cheese showed the highest ability to produce β-gal. It was identified and the sequence was deposited to the GenBank as *Bacillus Licheniformis* 17KAN-M3 under accession numbers OR742338. Optimization by one- variable -at-a-time showed 3555 U/ml of β-gal activity which is 1.60- fold higher as compared to un-optimized conditions. The CCd optimization lead to increase β-gal production from 6278 U/ml under PBd to 14048 U/ml (trial 19), resulting in a 2.24-fold increase in β-gal production. After applying the optimization methods, an overall increase of 6.26-fold in β-gal production was achieved.

Acknowledgment

Authors would like to acknowledge Prof. Faten Ahmed Ali (Chemistry of Natural and Microbial Products Department, National Research Centre) for her assistance in the statistical experiments.

Conflicts of interests

The authors declare that there are no conflicts of interests.

References

1. Abdaltef, M.J., Al-Temimi, W.K., Al-Shawi, S.G.; Extraction of β -galactosidase enzyme from Lacticacid bacteria. 4th International Conference of Modern Technologies in Agricultural Sciences. IOP Conference Series: Earth and Environmental Science, 1262, 062018, 2023.
2. Yan, Y., Guan, W., Li, X., Gao, K., Xu, X., Liu, B., Zhang, W., Zhang, Y.; β-galactosidase GALA from *Bacillus circulans* with high transgalactosylation activity, Bioengin., 12(1), 8908-8919, 2021.
3. Kaur, M., Sood, A., Chauhan, G.S., Gupta, R.; β-galactosidase: A potential biotechnological enzyme, Trends Carbohydr Res., 14 (4), 26-39, 2023.
4. Vasudha, M., Gayathri, D.; Kinetic and modeling analyses of lactose- hydrolyzing β-galactosidase from *Lactiplantibacillus plantarum* GV54. World Acad Sci J., 5 (11), 2023.
5. Abdollahi,S., Raoufi, Z.; Enhancing β-galactosidase production via GAL80 gene knockout in *Kluyveromyces marxianus*; an in-vitro and in-silico study on GAL/LAC system proteins. Food Biosci., 61, 104812, 2024.

6. Duan, F., Zhao, R., Yang, J., Xiao, M., Lu, L.; Integrated utilization of dairy whey in probiotic β-galactosidase production and enzymatic synthesis of galacto-oligosaccharides. Catalyst., 11, 658, 2021.
7. Anbalagan, N., Vaithilingam, M.; An experimental study using the statistical tool D-optimal design for the process optimization to enhance the yield of β-galactosidase from *Lactobacillus plantarum*. IJPER., 55(4), 1096-1106, 2021.
8. Abdel Wahab, W.A., Ahmed, S.A., Kholif, A.M.M., Abd El Ghani, S., Wehaidy, H.R.; Low-lactose yoghurt production using β-galactosidase: An integrated study for the enzyme and its application Int. Dairy J., 151, 105864, 2024.
9. Singh, R.V., Sambyal, K.; β- galactosidase as an industrial enzyme: production and potential. Chemical Papers, 77:11–31, 2023.
10. Wehaidy, H.R., Abdel-Naby, M.A., Kholif, A.M.M., Elaaser, M. Bahgaat, W.K., Abdel Wahab, W.A.; The catalytic and kinetic characterization of Bacillus subtilis MK775302 milk clotting enzyme: comparison with calf rennet as a coagulant in white soft cheese manufacture Journal of Genetic Engineering and Biotechnology, 21 (1), 61, 2023.
11. Wehaidy, H.E., Wahab, W.A.A., Kholif, A.M.M., Elaaser, M., Bahgaat, W.K ., Abdel-Naby, M.A., Statistical optimization of *B. subtilis* MK775302 milk clotting enzyme production using agro-industrial residues, enzyme characterization and application in cheese manufacture, Biocatalysis and Agricultural Biotechnology 25, 101589, 2020.
12. Coelho, R.J.S., Gabardo, S., Marim, A.V.C., Bologesi, L.S., Filho, N.J.P., Ayub, M.A.Z.; Porungo cheese whey: a new substrate to produce β-galactosidase. An Acad Bras Cienc., 95(4): e20200483, 2023.
13. Chakraborti, S., Sani, R.K., Sahoo, D.K., Banerjee, U.C., Sobti, R.C.; Production and partial characterization of a novel β-galactosidase from a newly isolated *Bacillus polymyxa*. Sci Iran., 10(3), 279-286, 2003.
14. Hsu, C. A., Yu, R. C., Chou, C. C., Production of b-galactosidase by Bifidobacteria as Influenced by various culture conditions. International Journal of Food Microbiology, 104, 197e206. 2005.
15. Deng, Y., Xu, M., Dawei, J., Agyei, D.; Optimization of β-galactosidase production by batch cultures of *Lactobacillus leichmannii* 313 (ATCC 7830™). Ferment., 6(27), 2020.
16. Akcan, N.; Cultural conditions optimization for production of β-galactosidase from *Bacillus licheniformis* ATCC 12759 under solid-state fermentation. Turk J Biochem., 43(3), 240-247, 2018.
17. R.L. Plackett, J. P. Burman, The design of optimum multifactorial experiments. Biometrika. 33, 305–325, 1946.
18. Box, G.E.P., Wilson, K.B.; On the experimental attainment of optimum conditions. Journal of the Royal Statistical Society: Series B (Methodological), 13, 1–38, 1951.
19. Kalathinathan, P., Muthukaliannan, G.K.; A statistical approach for enhanced production of β-galactosidase from *Paracoccus* sp. and synthesis of galacto-oligosaccharides. Folia Microbiol., 65, 811-822, 2020.
20. Alikunju, A.P., Joy, S., Rahiman, M., Rosmine, E., Antony, A.C., Solomon, S., Manjusha, K., Saramma, A.V., Krishnan, K.P., Hatha, A.M.; A statistical approach to optimize cold active β-galactosidase production by an arctic sediment pscychrotrophic bacteria, *Enterobacter ludwigii* (MCC 3423) in cheese whey. Catal Letters, 148(2), 712–724, 2018.
21. Amin AA, Olama ZA and Ali SM , Characterization of an isolated lactase enzyme produced by Bacillus licheniformis ALSZ2 as a potential pharmaceutical supplement for lactose intolerance. Front. Microbiol. 14:1180463, 2023.
22. Vasudha, M., Gayathri, D.; Kinetic and modeling analyses of lactose- hydrolyzing β- galactosidase from *Lactiplantibacillus plantarum* GV54. World Acad Sci J., 5(2), 1-7, 2023.