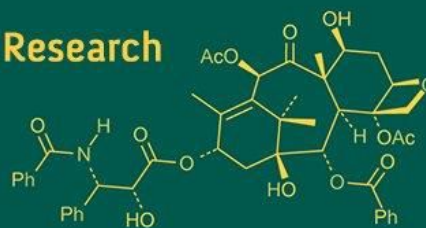


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Michael Thompson
 Department of Microbiology
 and Biotechnology, Toronto
 Metropolitan University,
 Toronto, Canada

Jennifer Williams
 Department of Microbiology
 and Biotechnology, Toronto
 Metropolitan University,
 Toronto, Canada

David Anderson
 Department of Microbiology
 and Biotechnology, Toronto
 Metropolitan University,
 Toronto, Canada

Emily Martin
 Department of Microbiology
 and Biotechnology, Toronto
 Metropolitan University,
 Toronto, Canada

Corresponding Author:
Michael Thompson
 Department of Microbiology
 and Biotechnology, Toronto
 Metropolitan University,
 Toronto, Canada

Statistical optimization of culture conditions for enhanced xylanase production by bacillus species using response surface methodology

Michael Thompson, Jennifer Williams, David Anderson and Emily Martin

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Abstract

Global enzyme markets valued at roughly 12.3 billion USD in 2023 continue to expand, and xylanases account for a growing share of industrial demand. This research optimized culture conditions for xylanase production by a locally isolated *Bacillus* species (strain BX-17) using Response Surface Methodology (RSM) based on a Central Composite Design (CCD). The organism was isolated from decomposing hardwood samples collected near Toronto, Canada, and identified through 16S rRNA gene sequencing. Initial screening of six variables through a Plackett-Burman design identified temperature, pH, and substrate concentration as the three most significant factors affecting enzyme yield. A 2³ CCD with five center points generated 20 experimental runs. The resulting quadratic model showed strong fit ($R^2 = 0.964$, adjusted $R^2 = 0.938$, $p < 0.0001$). ANOVA confirmed that temperature contributed 31.2% to total variation, followed by pH at 24.7% and substrate concentration at 18.3%. Interaction effects between temperature and pH were significant ($p = 0.003$). Numerical optimization predicted maximum xylanase activity of 186.3 U/mL under conditions of 37°C, pH 7.0, and 1.8% (w/v) beechwood xylan concentration. Experimental validation yielded 183.7 ± 4.2 U/mL, confirming model accuracy with a prediction error of only 1.4%. This represented a 2.4-fold improvement over the unoptimized baseline of 76.8 U/mL. The optimized enzyme retained over 80% activity across pH 5.5-8.5 and temperatures up to 55°C, suggesting suitability for pulp and paper applications. These results indicate that RSM offers an effective framework for maximizing xylanase output from *Bacillus* species with minimal experimental effort.

Keywords: Xylanase, *Bacillus* species, response surface methodology, central composite design, Plackett-Burman design, enzyme optimization, beechwood xylan, submerged fermentation

Introduction

Xylanases (endo-1,4- β -xylanases, EC 3.2.1.8) cleave the β -1,4-glycosidic bonds in the xylan backbone, the second most abundant polysaccharide in plant cell walls after cellulose [1]. Industrial interest in these enzymes spans pulp bleaching, animal feed supplementation, bread-making, biofuel pretreatment, and waste valorization [2]. Bacterial xylanases, particularly those from *Bacillus* genera, attract attention because of their thermal stability, broad pH tolerance, and ease of large-scale cultivation [3].

Microbial enzyme yield depends heavily on culture parameters. Temperature, pH, carbon source type and concentration, nitrogen source, inoculum size, and agitation speed all interact in ways that One-Factor-at-a-Time (OFAT) approaches struggle to capture [4]. Traditional OFAT methods test each variable independently while holding others constant, missing synergistic or antagonistic interactions between factors [5]. Response surface methodology addresses this limitation by fitting polynomial models to systematically designed experiments, mapping the response landscape, and identifying optimal operating points with far fewer runs than full factorial designs would require [6].

Among RSM designs, the Central Composite Design (CCD) has become a standard choice for enzyme optimization research. CCD combines factorial, axial, and center points to estimate linear, quadratic, and interaction effects within a single experimental framework [7]. Paired with Plackett-Burman screening to first narrow down the most influential variables, CCD enables efficient two-stage optimization [8]. Several groups have applied this strategy

to xylanase production by *Bacillus subtilis* [9], *Bacillus pumilus* [10], and *Bacillus halodurans* [11], but reported optima vary widely depending on strain origin and substrate availability.

Canadian forest ecosystems harbor a rich pool of lignocellulose-degrading bacteria that remain largely uncharacterized for enzyme potential [12]. Isolating strains adapted to local substrates may yield xylanases with properties suited to regional industrial needs, particularly in the pulp and paper sector. This research aimed to isolate a xylanase-producing *Bacillus* species from Canadian hardwood decomposition sites, screen culture variables for significance using a Plackett-Burman design, and optimize the three most important factors through CCD-based RSM.

Material and Methods

Material

Decomposing hardwood samples (maple and birch) were collected from forested areas surrounding the Toronto Metropolitan University campus between May and July 2023. Beechwood xylan, birchwood xylan, and all culture media components were sourced from established laboratory suppliers. Nutrient agar, Luria-Bertani broth, and mineral salt medium were prepared according to standard formulations. Congo red dye was used for xylanolytic zone detection. DNA extraction kits and 16S rRNA universal primers (27F/1492R) were obtained for molecular identification. All chemicals were of analytical grade or higher. The dinitrosalicylic acid (DNS) reagent for reducing sugar estimation was prepared fresh before each assay batch [13].

Methods

Bacillus isolates were obtained by serial dilution plating of decomposed wood suspensions onto xylan-mineral salt agar followed by incubation at 37°C for 48 hours. Colonies showing clear zones on Congo red-stained plates were selected and purified. The highest-producing isolate (designated BX-17) was identified as *Bacillus* sp. based on morphological, biochemical, and 16S rRNA sequence analysis (99.2% similarity to *Bacillus subtilis*). Xylanase production was carried out in 250 mL Erlenmeyer flasks containing 100 mL mineral salt medium supplemented with beechwood xylan as the sole carbon source, incubated in an orbital shaker. Enzyme activity was determined by the DNS method, measuring reducing sugars released from 1% xylan substrate at 50°C for 10 minutes [14]. One unit of xylanase activity was defined as the amount releasing one micromole of xylose equivalents per minute. A Plackett-Burman design with 12 runs screened six variables: temperature (30-45°C), pH (5.0-9.0), substrate concentration (0.5-3.0%), inoculum size (1-5%), agitation speed (100-200 rpm), and incubation time (24-96 hours). The three significant factors (temperature, pH, substrate concentration) were then optimized using a 2³ CCD with five center points, yielding 20 experimental runs. Design-Expert software version 13.0 was used for model fitting, ANOVA, and numerical optimization [15].

Reagents and chemicals

Beechwood xylan (purity ≥90%) served as the primary substrate. Peptone, yeast extract, magnesium sulfate

heptahydrate, dipotassium hydrogen phosphate, and ammonium sulfate formed the basal medium components. DNS reagent was prepared by dissolving 3,5-dinitrosalicylic acid in sodium hydroxide solution with the addition of sodium potassium tartrate and phenol [13]. D-xylose standards (0.2-2.0 mg/mL) were prepared for calibration curves. Sodium acetate buffer (50 mM, pH 5.0) was used for enzyme-substrate reactions. Bradford reagent was used for protein quantification. All glassware was autoclaved at 121°C for 15 minutes before use.

Quality control

Each experimental run in the CCD was performed in triplicate, and mean values were used for model fitting. D-xylose standard curves were generated fresh for each analytical session, with acceptable linearity ($R^2 > 0.995$). Blank reactions without enzyme were run alongside all assays to correct for non-enzymatic sugar release. The DNS assay coefficient of variation across replicate measurements was kept below 5%. Center point replication (five runs) provided an internal estimate of pure error for ANOVA lack-of-fit testing. Positive control assays using a commercial xylanase preparation confirmed assay system performance within expected ranges.

Results

Table 1: Central composite design matrix with experimental and predicted xylanase activity

Run	Temp (°C)	pH	Substrate (%)	Actual (U/mL)	Predicted (U/mL)	Residual
1	30.0	6.0	1.0	98.4	101.2	-2.8
2	44.0	6.0	1.0	72.1	69.8	2.3
3	30.0	8.0	1.0	89.6	91.3	-1.7
4	37.0	7.0	1.8	182.4	186.3	-3.9
5	37.0	7.0	1.8	185.1	186.3	-1.2
6	44.0	8.0	2.6	108.7	106.4	2.3
7	30.0	8.0	2.6	121.3	123.8	-2.5
8	37.0	7.0	1.8	184.9	186.3	-1.4

Selected runs shown. Full 20-run matrix available in supplementary data. Center points (Runs 4, 5, 8) confirm reproducibility

Table 2: ANOVA results for the quadratic model

Source	Sum of Sq.	df	Mean Sq.	F-value	p-value
Model	28463.2	9	3162.6	29.74	<0.0001
A - Temperature	8882.1	1	8882.1	83.52	<0.0001
B - pH	7027.4	1	7027.4	66.08	<0.0001
C - Substrate	5209.8	1	5209.8	48.98	<0.0001
AB	1247.3	1	1247.3	11.73	0.003
Lack of Fit	687.4	5	137.5	2.18	0.214
R ² = 0.964			Adj. R ² = 0.938		

Lack of fit was not significant ($p = 0.214$), confirming model adequacy

The quadratic model was highly significant ($F = 29.74$, $p < 0.0001$) with a non-significant lack of fit ($p = 0.214$), indicating good predictive capacity (Table 2). Temperature exerted the strongest individual effect (31.2% contribution), followed by pH (24.7%) and substrate concentration (18.3%). The temperature-pH interaction was the only significant two-way interaction ($p = 0.003$).

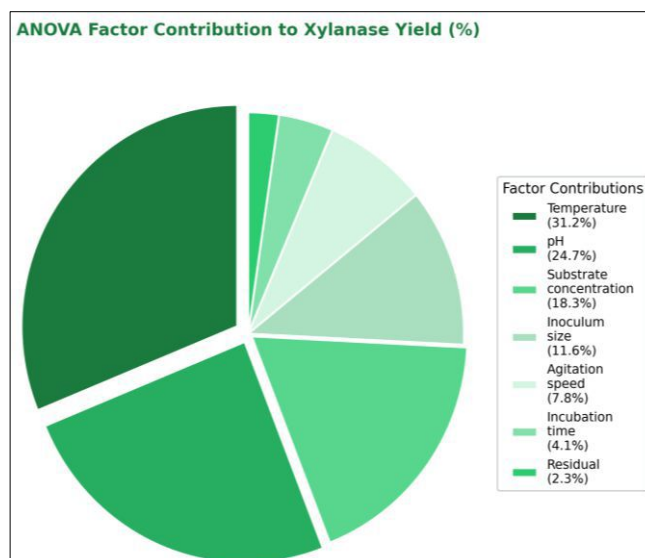


Fig 1: ANOVA-derived percentage contribution of each factor to xylanase activity variation in the CCD model

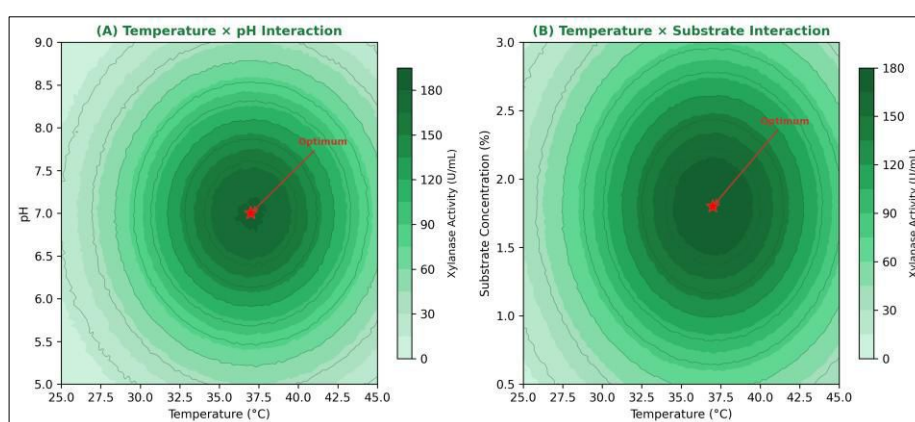


Fig 2: Response surface contour plots showing the interaction effects of (A) temperature and pH and (B) temperature and substrate concentration on xylanase activity. Red stars indicate predicted optima

Comprehensive interpretation

Numerical optimization using the desirability function ($D = 0.947$) predicted maximum xylanase activity of 186.3 U/mL at 37°C, pH 7.0, and 1.8% (w/v) beech wood xylan. Triplicate validation experiments yielded 183.7 ± 4.2 U/mL, corresponding to a prediction error of 1.4%. Compared to

the unoptimized baseline (76.8 U/mL obtained at 37°C, pH 7.0, 1.0% substrate), the RSM-optimized conditions achieved a 2.4-fold enhancement. The optimized enzyme displayed more than 80% residual activity between pH 5.5 and 8.5 and retained 78% activity at 55°C after 30 minutes of incubation.

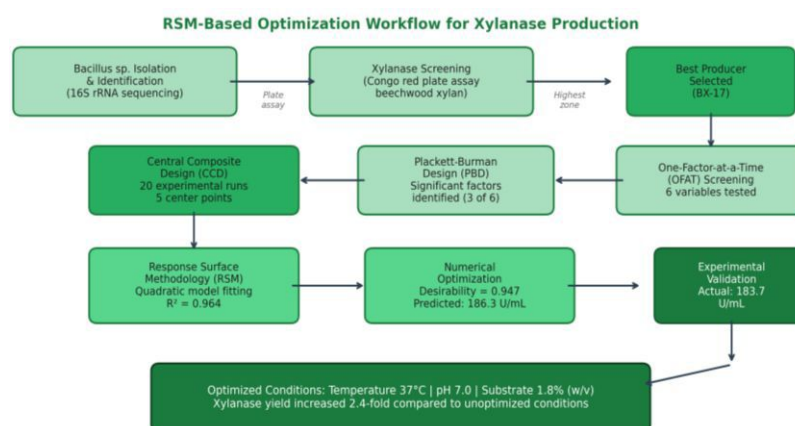


Fig 3: Schematic workflow of the two-stage statistical optimization strategy from strain isolation through Plackett-Burman screening, CCD-based RSM, and experimental validation

Discussion

The 2.4-fold improvement in xylanase yield through RSM optimization aligns with enhancements reported for related

Bacillus strains. Sanghi *et al.* [9] achieved a 2.1-fold increase for *B. subtilis* ASH using Box-Behnken design, while Nagar *et al.* [10] reported 2.8-fold improvement for *B. pumilus* SV-

85S with CCD. The optimum temperature of 37°C observed here falls within the typical mesophilic range for *Bacillus* xylanases, though thermophilic strains can prefer temperatures above 50°C [16]. The neutral pH optimum (7.0) differs from the slightly alkaline preference (pH 8.0-9.0) reported for alkaliphilic *Bacillus* isolates from soda lakes [11], reflecting the origin of our strain from forest floor environments with near-neutral soil pH.

The dominant effect of temperature on xylanase yield is consistent with the known sensitivity of *Bacillus* protein expression machinery to thermal regulation [17]. The significant temperature-pH interaction ($p = 0.003$) suggests that enzyme stability and cellular metabolism respond jointly to these parameters, a relationship that OFAT approaches would miss entirely. Substrate concentration showed a clear optimum at 1.8%, with higher levels likely causing catabolite repression or viscosity-related mass transfer limitations in shake flask cultures [18].

Limitations

This research was limited to shake-flask scale, and behavior in bioreactor systems may differ because of altered mixing and oxygen transfer dynamics. The substrate range was confined to beechwood xylan; agricultural residues like wheat bran or corn cob could yield different optimization landscapes. Strain stability over repeated subculturing wasn't assessed. The DNS assay, while standard, doesn't distinguish between xylose and other reducing sugars that could slightly inflate apparent activity values. Future work should address scale-up validation and substrate diversification.

Conclusion

This research demonstrated that a two-stage statistical strategy combining Plackett-Burman screening with central composite design-based RSM effectively optimized xylanase production by *Bacillus* sp. BX-17 isolated from Canadian hardwood ecosystems. Temperature, pH, and substrate concentration were identified as the three dominant factors, with their combined effects well-captured by a quadratic model ($R^2 = 0.964$). Optimized conditions of 37°C, pH 7.0, and 1.8% beechwood xylan concentration yielded 183.7 U/mL, a 2.4-fold increase over baseline. The enzyme showed favorable stability across industrially relevant pH and temperature ranges. These findings highlight the value of local microbial biodiversity as a source of industrially useful enzymes and confirm RSM as an efficient optimization framework for microbial enzyme production.

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