Agric Res J **60** (1) : 91-97, *February* 2023
DOI No. 10.5958/2395-146X.2023.00015.7

OPTIMIZATION OF XYLANASE PRODUCTION FROM *ASPERGILLUS Spp.* HAU-F-6 USING PLACKETT BURMAN SCREENING DESIGN AND PATH OF STEEPEST ASCENT IN SUBMERGED FERMENTATION

Ajay Pal[1]* and Vinod Saharan[2]

[1]*Department of Biochemistry, College of Basic Sciences and Humanities, CCS Haryana Agricultural University, Hisar - 125004*

[2]*Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, Rajasthan, India*

ABSTRACT

Xylanases are mainly responsible for the hydrolysis of β-1,4 bonds in plant xylan, the main component of hemicelluloses. In the present investigation, statistical designs were successfully used to optimize the fermentation parameters for enhanced xylanase production from *Aspergillus spp.* HAU-F-6 in submerged cultivation. The initial carbon screening test found wheat bran as the best carbon source to replace the expensive xylan in the fermentation medium. In Plackett-Burman Screening Design (PBSD), eleven independent variables *viz.* wheat bran, KH_2PO_4, tween-80, calcium chloride, urea, yeast extract, ammonium sulphate, peptone, sodium carbonate, ferrous sulphate and magnesium sulphate were selected. Supplementation of high levels of wheat bran, tween-80, yeast extract, ammonium sulphate, $MgSO_4$, and $FeSO_4$ in the growth medium positively affected the xylanase production. Contrarily, KH_2PO_4, calcium chloride, urea, peptone and sodium carbonate at their low concentrations of 0.5, 0.25, 1.0, 3.0 and 0.5 g/l, respectively resulted in higher xylanase production. Yeast extract, ammonium sulphate, $MgSO_4$ and wheat bran had significant positive effects at 10% level. Analysis of variance of PBSD showed the model used for prediction to be significant ($p<0.10$). The levels of $CaCl_2$, yeast extract and ammonium sulphate were further optimized using the Path of Steepest Ascent (POSA). Compared with the basal medium, an overall 9.76 fold higher enzyme activity was achieved from the model of the optimized medium, and xylanase activity was found to be 20.8 IU/ml.

Keywords: Biomass, Lignocelluloses, PBSD, POSA, Optimization, Xylanase.

Plant cell wall composition varies by species and is dependent on cell type and developmental stage. Usually, 90% of a typical cell wall comprises cellulose, pectin, and hemicellulose (Butt *et al*., 2008). Cellulose is the principal constituent of cell wall polysaccharides and consists of a linear chain of β-1,4-linked D-glucose residues. Hemicellulose, after cellulose, is the next vital component of the cell wall and serves as a storage polymer in seeds (Evstatieva *et al*., 2010). Along with pectin polysaccharides and lignin, it interacts with cellulose fibrils to create a rigid structure that strengthens the plant cell wall. Hemicelluloses are composed of diverse polymers of pentoses, hexoses and uronic acids and have been effectively used to produce numerous functional molecules, including medicines, fuels and feeds. Among all the hemicelluloses, the most abundant is a linear polymer of β-1,4-linked D-xylose residues having distinct branches (Shallom and Shoham, 2003; Gupta *et al*., 2009). It is a biodegradable polymer that needs the activity of a few enzymes, including xylanases (EC 3.2.1.8).

*Corresponding author : ajaydrdo@rediffmail.com
Date of receipt: 17.09.2021, Date of acceptance: 28.02.2023

Microbial xylanases have also shown great promise in various industrial processes at different processing stages. They degrade xylan by hydrolytic activity to produce industrially important smaller molecules (Badhan *et al*., 2007). In recent years, interest in xylanases has markedly increased due to their potential applications in food and beverage sectors (bakery goods, coffee, starch, plant oil and juice manufacture), feedstock improvement (increasing animal feed digestibility) and the quality improvement of lignocellulosic residues (Bakri *et al*., 2008, Sharma *et al*., 2020).

Extracellular xylanases are commercially derived from filamentous fungi because they can quickly grow and produce high enzyme titers. *Aspergillus* and *Trichoderma* spp. are most commonly used at the industrial scale, where they are grown on solid and liquid media via solid-state-fermentation (SSF) and submerged fermentation (SmF), respectively (Nair *et al*., 2008; Ravindran *et al.,* 2019). These fermentation systems, which are closer to the natural habitats of microbes, prove more efficient in producing certain enzymes/metabolites. Physiological and biochemical factors such as temperature, pH, incubation time,

agitation and nutritional source of microbial strains significantly affect the production levels of enzymes/ metabolites. However, the economic profitability of enzyme/metabolite production depends on the cost and availability of the substrate and producer organism and the efficiency of the downstream processing (Becerra and Sisco, 1996). Usually, the 'one-factor-at-a-time' approach is used to optimize the growth conditions, but it is inherently bound to the missing of interactive effects among media components. However, response surface methodology (RSM) is routinely employed to solve such issues (Houng *et al*., 1989; Pal and Khanum, 2010; Ahmed *et al*., 2021). Our group is heavily engaged in the studies on industrially important microbial proteins (Pal *et al*., 2009; Pal and Ramana, 2009, 2010; Pal *et al*., 2010a,b, 2013; Narwal *et al*., 2016a,b; Narwal *et al*., 2017). In continuation, the present study identifies factors influencing xylanase production from *Aspergillus spp.* HAU-F-6 using Plackett-Burman Screening Design (PBSD) followed by Path of Steepest Ascent (POSA) of Response Surface Methodology (RSM).

MATERIALS AND METHODS

Microorganism and chemicals

We have isolated twenty-seven microorganisms from soil samples collected near perishables debris in Hisar. One of the hyper-secretor organisms was subjected to morphological test and identified as *Aspergillus spp.* and given the title *Aspergillus spp.* HAU-F-6. The isolate was grown for five days at 30°C on the slant of Potato Dextrose Agar (PDA) medium, and it was then shifted at 4°C. To perform the experiment, spores were harvested and inoculum was prepared in 0.1% tween-80 (autoclaved). The final count was adjusted to ~$1x10^5$ spores/ml. The chemicals used throughout the investigation were of high standard analytical grade.

Optimization of media

Effect of carbon source

Xylan is a costly component of potato dextrose broth commonly used for fungal growth. An attempt was made to replace it with various agro-industrial wastes like the peel of orange, corncob, bagasse of sugarcane and banana and pineapple peel. Wheat bran and oat bran, relatively cheaper sources, were also tried. Moreover, to ascertain the expression nature (inducible or constitutive) of xylanases, a few simple and directly usable forms of sugars (sucrose, maltose, dextrose, fructose, galactose, lactose, xylose) were also used.

Optimization procedure and experimental design

Next set of experiments was conducted using statistical strategies of RSM. Here, PBSD and POSA were used in tandem to get the maximum yield of xylanase.

1. Plackett-Burman Screening Design: The PBSD is frequently used to study/screen the effects of several independent variables on the dependent variables. Eleven independent variables (wheat bran, KH_2PO_4, Tween-80, calcium chloride, urea, yeast extract, ammonium sulphate, peptone, sodium carbonate, ferrous sulphate and magnesium sulphate) were screened in twelve combinations and one center point experiment was organized according to the design matrix (Tables 1 and 2). In this design, two levels i.e., high and low [denoted by (+) and (–), respectively] of each variable, are taken. The effect of each variable or factor is the difference between the averages of outcomes obtained at the high and low levels of the factor. The following equation calculates the same-

$$E_{(xi)} = 2(\sum P_{i+} - \sum P_{i-})/N$$

Where, $E_{(xi)}$ represents the concentration effect of the test variable. P_{i+} and P_{i-} represent enzyme activities from trials where the measured variable (xi) were present at high and low concentrations, respectively. N represents the number of experiments excluding the center point experiment.

This design screens the variables based on a first-order model:

$$Y = \beta_0 + \sum \beta_i X_i$$

Where, Y is the response (xylanase activity, IU/ml), β is the model intercept and β_i is the variable estimates (Plackett and Burman, 1946).

2. Path of Steepest Ascent: Based on the first-order model equation obtained from PBSD, the path of the steepest ascent was determined to find the proper direction of changing the variables. The design of the POSA is shown in Table 3.

Enzyme assay

The production medium, having enzyme secreted by the organism, was clarified by filtration through muslin cloth and centrifugation (10,000 X g for 20 min at 4°C). The obtained supernatant was subjected to activity assay using the earlier described method (Khanna, 1993). Briefly, the solution of xylan and enzyme at appropriate dilution was incubated at 37°C for 30 min. The reducing sugars were determined by dinitrosalicylic acid method as Miller (1959) described with xylose as standard. Xylanase hydrolyzed the substrate (xylan) to

produce xylose which was measured at 540nm using a spectrophotometer. Enzyme activity was presented as the international unit (1 IU = 1 µmol/min).

Data analysis

The obtained data were statistically analyzed using Design Expert and Minitab software.

RESULTS AND DISCUSSION

Optimization of medium

Effect of carbon source

Production of the enzyme is usually dictated by the carbon resource. Herein also, we found that the nature of carbon source in the production medium substantially affected the production and subsequent release in the medium. The highest enzyme titer was obtained when the medium was supplemented with wheat bran (2.98 IU/ml). It was followed by oat bran and sugarcane bagasse with the enzyme units 2.71 and 2.69 IU/ml, respectively. Supplementation of simple and relatively easy to utilize sugars did not support enzyme production (Fig. 1).

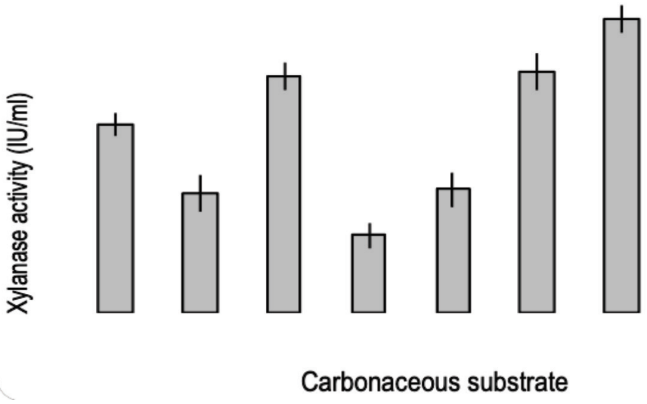

Fig. 1. Effect of carbon source on xylanase production

Results of the experiment suggest that enzyme production by the organism under investigation is inducible in nature. Earlier, many independent researchers have also studied this nature of enzyme where xylanase was not produced in the absence of xylan substrate (Coughlan and Hazlewood, 1993; Coughlan *et al.*, 1993). However, contrary to these observations, Laxmi *et al.* (2008) found that *Aspergillus* sp. RSP-6 produced and released xylanase in a constitutive manner. Wheat bran has also been reported as the best supporter of xylanase production by *A. niger* ANL 301 (Okafor *et al.,* 2007).

Optimization procedure and experimental design

After optimizing the carbon source, the next experiment was performed to standardize the other ingredients for xylanase production in SmF. It was accomplished using statistical designs namely, PBSD and POSA, used one after another. The PBSD is an essential tool in screening the effects of process variables on the yield. It can significantly reduce the number of repetitive experiments to be conducted in a further optimization study, using a response surface methodology (Ekpenyong *et al*., 2017).

In our strategy, 12 experiments were performed to screen the effects of 11 medium components. One center point experiment was also conducted in parallel as per the design matrix. The experimental data of xylanase production in the screening design experiments illustrated a wide variation in enzyme activity from 8.2 to 16.1 IU/ml, reflecting the importance of medium optimization to attain higher yields (Table 2). As described in materials and methods, calculations were made to decipher the main effect of each independent variable. In this set of experiments, the obtained data revealed a wide range of main effects that were positive in nature.

Table 1. Independent variables and their levels

Variable	Symbol	-1	0	+1
Wheat bran (g/l)	WB	5	10	15
KH_2PO_4 (g/l)	KH2	0.5	1.0	1.5
Tween-80 (ml/l)	T-80	0.1	0.2	0.3
Calcium chloride (g/l)	CaCl2	0.25	0.50	0.75
Urea (g/l)	U	1	2	3
Yeast extract (g/l)	YE	3	5	7
Ammonium sulphate (g/l)	AS	1	2	3
Peptone (g/l)	P	3	5	7
Sodium carbonate (g/l)	SC	0.5	1.0	1.5
Ferrous sulphate (g/l)	FeS	0.5	1.0	1.5
Magnesium sulphate (g/l)	MgS	0.5	1.0	1.5

Table 2. Experimental design matrix and results

WB	KH_2	T-80	$CaCl_2$	U	YE	AS	P	SC	FeS	MgS	Actual xylanase activity (IU/ml)	Predicted xylanase activity (IU/ml)
1	1	-1	1	1	-1	1	-1	-1	-1	1	11.69±0.41	11.62
1	1	-1	1	-1	-1	-1	1	1	1	-1	8.28±0.45	8.22
-1	1	-1	-1	-1	1	1	1	-1	1	1	15.88±0.48	15.82
1	-1	1	-1	-1	-1	1	1	1	-1	1	15.86±0.49	15.81
-1	-1	1	1	1	-1	1	1	-1	1	-1	9.68±0.45	9.61
1	-1	-1	-1	1	1	1	-1	1	1	-1	16.18±0.64	16.11
1	-1	1	1	-1	1	-1	-1	-1	1	1	15.25±0.62	15.21
-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	11.97±0.53	11.91
-1	1	1	-1	1	-1	-1	-1	1	1	1	11.18±0.48	11.12
0	0	0	0	0	0	0	0	0	0	0	12.88±0.45	12.55
-1	-1	-1	1	1	1	-1	1	1	-1	1	9.86±0.47	9.81
-1	1	1	1	-1	1	1	-1	1	-1	-1	12.98±0.55	12.92
1	1	1	-1	1	1	-1	1	-1	-1	-1	12.48±0.52	12.42

Findings indicate that high amounts of medium components, namely wheat bran, tween-80, yeast extract, ammonium sulphate, $MgSO_4$ and $FeSO_4$, positively supported xylanase production by strain. While the high levels of ingredients *viz.* KH_2PO_4, calcium chloride, urea, peptone and sodium carbonate were not desirable in the medium as their lowest levels were optimum for xylanase production (Table 3). Analysis of the different components showed that $CaCl_2$ (26.16%), yeast extract (20.54%) and ammonium sulphate (18.26%) showed a high-percentage contribution to the production of xylanase from the organism under study. The relative effects of studied independent variables on xylanase production are elucidated in the Pareto graph (Fig. 2). The t-value and p-value were calculated to explain the effect of each variable statistically. The main effects of yeast extract, ammonium sulphate, $MgSO_4$ and wheat bran were significant at 10%, demanding further standardization. Results of ANOVA for PBSD model are presented in Table 4. 'Model F-value' of 110.18 indicates that the model is significant at 0.10 α value. A 7.55 fold increase in enzyme activity (16.1 IU/ml) could be achieved using PBSD. The following regression equation was obtained using the PBSD, which predicts the factors that affected the response:

Xylanase activity (IU/ml): 12.554 + 0.683WB - 0.533KH_2 + 0.300T-80 - 1.317$CaCl_2$ - 0.767U + 1.167YE + 1.10AS - 0.60P - 0.217SC + 0.133FeS + 0.683MgS

The PBSD is a valuable tool for screening parameters that significantly affect the response, but it cannot predict the optimum region of the parameters. Based on the obtained first-order model equation and three important medium parameters, the steepest

Table 3. Statistical analysis of medium optimization using PBSD for xylanase production

Variable	Effect	% Contribution	Co-efficient	St. Dev.	t-value	*p*-value
WB	1.367	7.05	0.683	0.07396	9.24	0.069
KH_2	-1.067	4.29	-0.533	0.07396	-7.21	0.088
T-80	0.600	1.36	0.300	0.07396	4.06	0.154
$CaCl_2$	-2.633	26.16	-1.317	0.07396	-17.80	0.036
U	-1.533	8.87	-0.767	0.07396	-10.37	0.061
YE	2.333	20.54	1.167	0.07396	15.77	0.040
AS	2.200	18.26	1.100	0.07396	14.87	0.043
P	-1.200	5.43	-0.600	0.07396	-8.11	0.078
SC	-0.433	0.71	-0.217	0.07396	-2.93	0.209
FeS	0.267	0.27	0.133	0.07396	1.80	0.322
MgS	1.367	7.05	0.683	0.07396	9.24	0.069

Table 4. ANOVA for PBSD model

Source	DF	SS	MS	F-value	p-value
Main Effects	11	79.5067	7.22788	110.18	0.074
Residual Error	1	0.0656	0.0656		
Total	12	79.5723			

Tabulated F-value$_{(11, 1)}$ = 60.472 (α = 0.10)
Tabulated F-value$_{(11, 1)}$ = 242.983 (α = 0.05)

Table 5. Experimental results along the POSA

Items	X_1 ($CaCl_2$) (g/l)	X_2 (Yeast extract) (g/l)	X_3 (Ammonium sulphate) (g/l)	Actual xylanase activity (IU/ml)	Predicted xylanase activity (IU/ml)
Base point	0.50	5.0	2.0	-	
Original step size	0.25	2.0	1.0	-	
Slope	-1.317	+1.167	+1.100	-	
Range	-0.329	+2.334	+1.100	-	
New step size	-0.08225	0.5835	0.275	-	
Expt 1 (Base)	0.50	5.0	2.0	12.9	12.9
Expt 2 (Base+1Δ)	0.41775	5.5835	2.275	14.1	14.4
Expt 3 (Base+2Δ)	0.3355	6.167	2.550	15.8	16.1
Expt 4 (Base+3Δ)	0.25325	6.7505	2.825	17.6	17.2
Expt 5 (Base+4Δ)	0.171	7.334	3.1	20.8	20.5
Expt 6 (Base+5Δ)	0.08875	7.9175	3.375	19.1	19.4
Expt 7 (Base+6Δ)	0.0065	7.334	3.65	17.4	17.8

Table 6. Comparison of initial and final activity of xylanase

Organism	Fermentation Type	Stage	Activity (IU/ml)	Fold increase
Aspergillus spp. HAU-F-6	SmF	Initial	2.13	9.76
		Final	20.8	

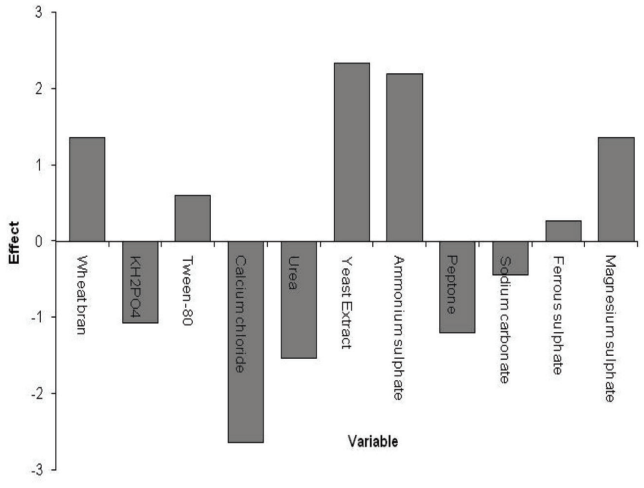

Fig. 2. Pareto graph showing the effect of variables

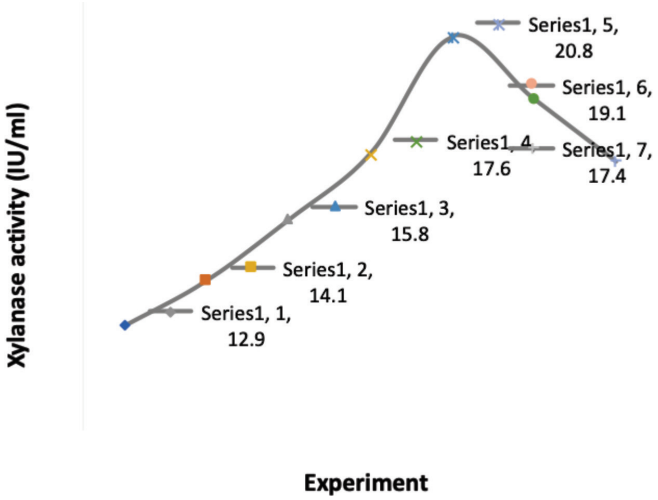

Fig. 3. Production of xylanase in different experiments of POSA

ascent method was applied to find an adequate direction of changing parameters. The way of the steepest ascent was determined to find the proper direction of changing variables by decreasing the concentration of $CaCl_2$ (X_1) and increasing the concentrations of yeast extract (X_2) and ammonium sulphate (X_3). Several experiments were conducted to locate the plateau of the response. The path of the steepest ascent and the obtained results are given in Table 5 and Fig. 3. It was found that maximum production (20.8 IU/ml) occurred in experiment number 5, providing an overall 9.76 fold increase in xylanase production (Table 6).

In conclusion, the most potent xylanase-producing fungal isolate, *Aspergillus spp.* HAU-F-6, was employed for xylanase production in SmF conditions. PBSD and POSA were explored to approach near the vicinity of the optimum composition of the medium. The optimized concentrations (g/l) of $CaCl_2$, yeast extract and ammonium sulphate were 0.171, 7.334 and 3.1, respectively and an overall 9.76 fold increase in xylanase production could be achieved.

Authors' contribution

Conceptualization, designing and execution of the research work (AP); Execution of experiments and data collection (AP, VS); Analysis of data and interpretation (VS); Preparation of the manuscript (AP, VS)

LITERATURE CITED

Ahmed T, Rana M R, Zzaman W, Ara R and Aziz M G 2021. Optimization of substrate composition for pectinase production from Satkara (*Citrus macroptera*) peel using *Aspergillus niger*-ATCC 1640 in solid-state fermentation. *Heliyon* **7**. e081332.

Badhan A K, Chadha B S, Kaur J, Saini H S and Bhat M K 2007. Production of multiple xylanolytic and cellulolytic enzymes by thermophilic fungus *Myceliophthora* sp. IMI 387099. *Bioresour Technol* **98**:504-10.

Bakri Y, Al-Jazairi M and Al-Kayat G 2008. Xylanase production by a newly isolated *Aspergillus niger* SS7 in submerged culture. *Pol J Microbiol* **57**(3):249-51.

Becerra M and Siso M G 1996. Yeast β-galactosidase in solid-state fermentations. *Enzyme Microb Technol* **19**(1): 39-44.

Butt M S, Tabir-Nadeem M, Ahmad Z and Sultan M T 2008. Xylanases and their application in baking industry. *Food Technol Biotechnol* **46**(1):22-31.

Ekpenyong M G, Antai S P, Asitok A D and Ekpo BO 2017. Plackett-Burman Design and Response Surface Optimization of medium trace nutrients for glycolipopeptide biosurfactant production. *Iran Biomed J* **21**(4):249-60.

Evstatieva Y, Nikolova D, Ilieva S, Getov L and Savov V 2010. Identification and characterization of α-amylase and endoxylanase, produced by *Aspergillus* mutant strains. *Biotechnol Biotechnol Equip* **24**:613-17.

Gupta V K, Gaur R, Gautam N, Kumar P, Yadav I J and Darmwal N S 2009. Optimization of xylanase production from *Fusarium solani* F7. *Am J Food Technol* **4**(1):20-29.

Houng J Y, Chen K C and Hsu WH 1989. Optimization of cultivation medium composition for isoamylase production. *Appl Microbiol Biotechnol* **31**:61–64.

Khanna S 1993. Regulation, purification and properties of xylanase from *Cellulomonas fimi. Enzyme Microb Technol* **15**:990-95.

Miller G L 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**:426-28.

Nair S G, Sindhu R and Shashidhar A 2008. Fungal xylanase production under solid state and submerged fermentation conditions. *Afr J Microbiol Res* 2008, **2**:82-86.

Narwal R K, Bhushan B, Pal A, Malhotra S P, Kumar S and Saharan V 2016a. Inactivation thermodynamics and iso-kinetic profiling for evaluating operational suitability of milk clotting enzyme immobilized in composite polymer matrix. *Int J Biol Macromol* **91**: 317–28.

Narwal R K, Bhushan B, Pal A, Panwar A and Malhotra S 2016b. Purification, physico-chemico-kinetic characterization and thermal inactivation thermodynamics of milk clotting enzyme from *Bacillus subtilis* MTCC 10422. *LWT- Food Sci Technol* **65**: 652-60.

Narwal R K, Bhushan B, Pal A and Malhotra S P 2017. Optimization of upstream process parameters for enhanced production of thermostable milk clotting enzyme from *Bacillus subtilis* MTCC 10422. *J Food Process Eng* **40**(2).e12356.

Pal A and Ramana K V 2009. Isolation and preliminary characterization of a nonbacteriocin antimicrobial compound from *Weissella paramesenteroides* DFR-8 isolated from cucumber (*Cucumis sativus*). *Process Biochem* **44**: 499-503.

Pal A and Khanum F 2010. Production and extraction optimization of xylanase from *Aspergillus niger* DFR-5 through solid-state-fermentation. *Bioresour Technol* **101**: 7563-69.

Pal A and Ramana K V 2010. Purification and characterization of bacteriocin from *Weissella paramesenteroides* DFR-8, an isolate from cucumber (*Cucumis sativus*). *J Food Biochem* **34**: 932-48.

Pal A, Lobo M and Khanum F 2013. Extraction, purification and thermodynamic characterization of almond (*Amygdalus communis*) β-galactosidase for the preparation of delactosed milk. *Food Technol Biotechnol* **51**(1): 53-61.

Pal A, Pal V, Ramana K V and Bawa A S 2009. Biochemical studies of β-galactosidase from *Kluyveromyces lactis. J Food Sci Technol* **46** (3): 217-20.

Pal A, Ramana K V and Bawa A S 2010a. Simplification and optimization of MRS medium for enhanced production of bacteriocin from DFR-8. *J Food Sci Technol* **47** (3): 254-61.

Pal V, Pal A, Patil M, Ramana K V and Jeevaratnam K 2010b. Isolation, biochemical properties and application of bacteriocins from *Pediococcus pentosaceous* isolates. *J Food Process Preserv* **34**: 1064-79.

Plackett R L and Burman J P 1946. The design of optimum multifactorial experiments. *Biometrika,* **33**: 305-25.

Ravindran R, Williams G A and Jaiswal A K 2019. Spent coffee waste as a potential media component for xylanase production and potential application in juice enrichment. Foods **8**(11):585.

Shallom D and Shoham Y 2003. Microbial hemicellulases. *Curr Opin Microbiol* **6**:219-28.

Sharma S, Sharma V, Nargotra P and Bajaj B K 2020. Bioprocess development for production of a process-apt xylanase with multifaceted application potential for a range of industrial processes. *SN Appl Sci* **2**(4):1-15.