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Screening of agro-industrial waste and physical factors for the optimum production of pullulanase in solid-state fermentation from endophytic *Aspergillus* sp.

Bindu Naik [a,*], S.K. Goyal [a], A.D. Tripathi [b], Vijay Kumar [c]

[a] Department of Farm Engineering, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, 221005, Uttar Pradesh, India
[b] Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, 221005, Uttar Pradesh, India
[c] Department of Biosciences, Swami Rama Himalayan University, Jolly Grant, Dehradun, Uttarakhand-248140-India

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ABSTRACT

The selection of a suitable agricultural residue, its availability and the cost are critical factors to be considered while selecting a raw material in solid-state fermentation (SSF). In the case of SSF parameters like temperature, moisture content and spore concentration also play a significant role in enzyme production. In this study, endophytic fungi were isolated and screened for their pullulanase activity. The most promising isolate identified as *Aspergillus* sp. was evaluated for the pullulanase production using agricultural residual substrates like wheat bran, rice bran, sugarcane bagasse, orange peel, mosambi peel, and banana peel. Among all the substrates tested by the classical approach, the wheat bran was found to be the most effective substrate. The highest yield was 65.33 ± 2.08U/gds, while in case of rice bran the maximum yield was 44.66 ± 1.527U/gds. The comparatively lower yield was obtained in sugarcane bagasse, orange peel, mosambi peel, and banana peel. Maximum Pullulanase activity (67.00 ± 01.00 U/gds) was reached on 3rd day; thereafter the enzyme activity declined. Experiments conducted at the optimum physical conditions (temperature 28.62 °C; moisture 69.92%; inoculum size 6.42 log) demonstrated that the pullulanase yield (396.2 ± 1.33U/gds) was closer to the predicted value (394.5U/gds). There was an improvement of yield by 6.064 fold relative to that obtained from un-optimized physical parameters. The good correlation between predicted and experimental values after optimization justified the validity of the response model and the existence of an optimum point. This is the first report of pullulanase production from *Aspergillus* species in solid-state fermentation.

1. Introduction

Pullulanases constitutes an important group of industrial enzyme which belongs to a family of glycosyl hydrolases 13, also called as the α-amylase family (Henrissat, 1991). They hydrolyze the glycosidic bonds in the starch during the saccharification process which leads to the production of glucose, maltose, and maltotriose syrups. These products are widely used in the food and other related industries. Among the starch hydrolyzing enzymes, pullulanase possess both α-1,6 and/or α-1,4 hydrolysis activity on pullulan as well as other carbohydrates such as starch, amylopectin, and glycogen (Domań Pytka and Bardowski, 2004; Hii et al., 2009). The enzyme in combination with α-glucosidases give a better result and enhance the rate of starch saccharification increasing the reducing sugars yield. This unique feature makes it more important for industrial applications than the other hydrolases. Only a limited study has been reported on the production of pullulanase with native strains of fungi because its productivity is too low. In line with the increasing demands for pullulanase, it has become important to search for novel pullulanase-producing microorganisms with high yields. Moreover, high production cost and low yield are major limitations in the industrial production of pullulanase enzyme. The production cost of pullulanase can be minimized by selecting agro-industrial waste as the substrate for the enzyme production under solid-state fermentation (SSF) processes. A number of such agro-industrial wastes are sugar cane bagasse, wheat bran, rice bran, maize bran, green gram husk, wheat straw, rice straw, rice husk, soyhull, sago hampas, grapevine trimmings dust, sawdust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil

* Corresponding author.
E-mail address: binnaik@gmail.com (B. Naik).

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mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, cornflour, steamed rice, steam pretreated willow, starch, etc. Wheat bran, however, holds the key, and has most commonly been used, in various processes (Pandey et al., 1999; Rosalese et al., 2002). The use of these agricultural wastes in bioprocesses may provide alternative substrates and furthermore, helps to solve environmental problems as well as reduce the production cost of enzymes. So, in this study, this limitation will be trying to overcome in order to find potential pullulanase producing microorganisms and cheap substrates which will be suitable for the production and increased yield of pullulanase. As well as there are a very few reports on pullulanase from fungal sources. After having good industrial applications, only due to high cost pullulanase lack its industrial uses. On the basis of the above research, the present study was performed on Screening of the potential low cost agro-waste for the production of pullulanase in SSF and to optimize the physical parameters for the optimum production of the enzyme. Because these physical parameters are very important to enhance, promote and stimulate the optimum production of enzymes (Rahman et al., 2005). Also, the factors like time, moisture, temperature and spores (inoculum) optimization is very important for the successful production of enzymes (Lonsane et al., 1985; Lakshmi et al., 2009).

2. Materials and methods

2.1. Isolation and screening of endophytic fungi producing pullulanase

The fungal endophytes were isolated from the ornamental plant (*Tradescantia pallida)* collected from BHU Campus, Lanka, Varanasi, India. The isolation of endophytes was performed according to the method described previously (Bezerra et al., 2013). The isolates were screened for their ability to hydrolyze the pullulan by agar plate method (25 °C for 72 h). After 72 h of incubation at 25 °C, the Petri dishes were flooded with Gram's iodine and observed for the clear zone around the fungal colonies. In the secondary screening (Smf without agro-waste) the fungal strains were inoculated on Pullulan broth medium (1% pullulan, 0.5% $(NH_4)_2SO_4$, 0.5% KH_2PO_4, 0.2% NaCl, and 0.2% $MgSO_4$) and incubated at 25 °C for 72 h. The culture filtrate was used as an enzyme. The 1.5% agar was supplemented with 1% pullulan, autoclaved and poured into petri dishes (25 ml). Agar wells were made in pullulan agar and poured 50 μl of culture filtrate and incubate at 25 °C for 12 h. Its pullulanase activity was further confirmed by the thin layer chromatography (TLC) of the hydrolysed products. The solvent system consists of a mixture of chloroform, acetic acid, and water (3:3.5:0.5). The most promising isolate BHU-46 was identified according to the method described by Naik et al. (2017). The schematic representation of the study has been given in Fig. 1.

2.2. Culture conditions

The promising isolate BHU-46 was grown on Potato dextrose agar medium at 27 °C for 72 h for proper sporulation. Spore suspensions of fungi were used as the inoculum by adding 10 ml sterile distilled water and scraping an agar slant with the help of a loop. A standard inoculum of 1 ml in which the spore numbers were 1.2×10^4 CFU per mL, was received by each flask.

2.3. Substrate

For the solid-state fermentation (SSF) various agro-industrial residues such as Wheat bran (WB), rice bran (RB), sugarcane bagasse (SB), orange peel (OP) mousambi peel (MP), and banana peel (BP)

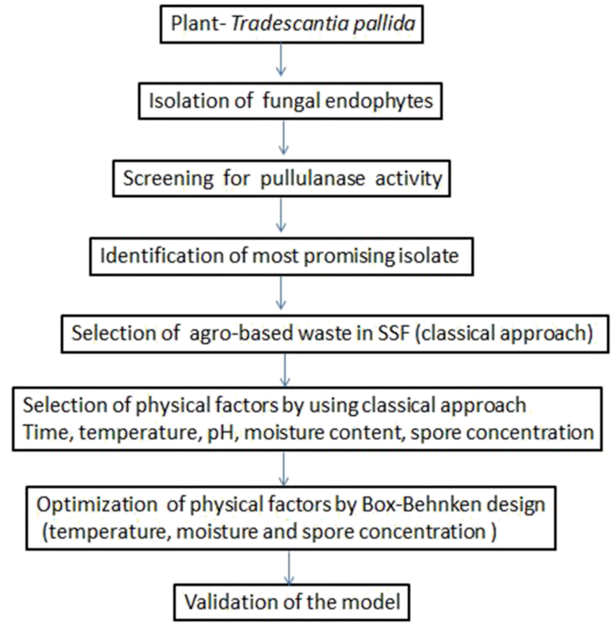

Fig. 1. The schematic representation of the study.

were screened for their potential as a substrate for the production of Pullulanase.

2.4. Preparation of inoculum and solid-state cultivation

Spore suspensions of fungi was used as the inoculum by adding 10 ml sterile distilled water and scraping an agar slant with the help of a loop. A standard inoculum of 1 ml in which the spore numbers were 1.2×10^4 CFU per ml, was received by each flask. Incubation of the culture flasks were done at 27 °C for 48 h.

2.5. Selection of suitable agro-waste by using classical approach in Smf and SSF

The SSF fermentation was carried in 250 ml flask containing 05 g (dry weight) powdered solid substrate supplemented with nutrient salts- 1% $(NH_4)_2SO_4$, 1% KH_2PO_4, 0.2% NaCl, and 0.2% $MgSO_4$. The flasks were sterilized at 121 °C for 20 min and, after cooling, inoculated with 1 mL of spore suspension followed by incubation at 27 °C for the 5 days. The initial moisture content was adjusted to 60%. All liquid added to the flask was taken into consideration in calculating the moisture content. Enzyme was extracted according to the method described previously sections.

Submerged fermentation (SmF) was carried out by taking 40 mL of the production medium containing nutrient salt as mentioned in SSF in 250 ml Erlenmeyer flasks. This was supplemented with various agro-industrial residues at 2% concentration. All the flasks were autoclaved at 121 °C for1 20 min and after cooling were inoculated with 1 ml of spore suspension and incubated on a rotary shaker at 180 rpm at 27 °C for the desired period. After fermentation, the supernatant was harvested by centrifugation at 10,000 g for 10 min (4 °C) and was used as crude enzyme extract.

2.6. Crude enzyme extraction

The crude enzyme was extracted by flooding the flasks with 50 mL of 1 mM of phosphate buffer (pH 6.5). This extraction step was for 1 h and at room temperature (35 °C), and then filtered by using a muslin cloth and funnel. The filtered extract was centrifuged in a cooling centrifuge at 4 °C for 15 min at 10,000 rpm so that all the cells and debris could be removed (Shamala and Sreekantiah, 1986). The supernatant was collected as the crude enzyme and pellets were discarded.

2.7. Enzyme activity and protein estimation

Pullulanase activity was assayed by the DNSA method (Miller, 1959). 0.1 ml of the extracted crude enzyme sample and 0.4 ml phosphate buffer (pH 6.5) were added to 0.5 ml of 1% (w/v) solution of starch. This mixture was incubated at 40 °C for 30 min 1 ml of DNS reagent was added and incubation of test tubes was done in a boiling water bath for 5–10 min. Then, after cooling down, 0.5 ml of 1% (w/v) Sodium Potassium Tartrate solution was also added. The final volume was adjusted to 5 ml by adding 2.5 ml of sterile distilled water. After this, the absorbance was measured at 570 nm. The protein estimation was performed according to the procedure of Lowry et al. (1951). One unit of a-amylase activity was defined as the amount of enzyme that produced reducing sugar equivalent to 1 μmole of glucose per min at 40○C temperature and at pH 6.5.

2.8. Selection of critical physical factors using classical approach

The effect of incubation time on enzyme production by the fungi was studied by incubating the inoculated flasks for a total period of 216 h and estimating the enzyme production at regular intervals of 24 h 5 g of wheat bran powder supplemented with nutrient salts- 1% $(NH_4)_2SO_4$, 1% KH_2PO_4, 0.2% NaCl, and 0.2% $MgSO_4$ was used in SSF. Other fermentation conditions were the same as mentioned above. The effect of moisture content was determined in range of 50–80% after 72 h. The effect of temperature on the production of enzyme was determined in the temperature range between 25 °C and 75 °C at 70% moisture after 72 h of incubation. Effect of spore concentration (1×10^4 to 1×10^9 spores/gds) on enzyme production was determined at 35 °C at 70% moisture, for 72 h. The effect of initial pH on enzyme yield by *Aspergillus* sp. BHU-46 during solid-state fermentation was studied by adjusting the pH of the mineral salt solution used to moisten the substrate to various pH levels (pH 5.0–7.5) using citrate phosphate buffer and fermentation was carried at 35 °C, spore concentration of 1×10^7 spores/gds.

2.9. Optimization of physical parameters using Box-Behnken design

The critical physical factors such as temperature, moisture and spore concentration significantly affected the production of Pullulanase (classical approach) was further optimized for three factors at three levels by Box-Behnken design (1960) using Design expert Software 10 as the method described previously by Francis et al. (2003).

3. Results and discussion

3.1. Isolation and screening of endophytic isolates producing pullulanase

A total of 51 endophytic fungi were recovered from *Tradescantia pallida,* out of which only one was found to produce the enzyme pullulanase. It was named as BHU-46 and was identified as *Aspergillus* sp. (Fig. 1S). Further the pullulanase activity was confirmed by TLC. It indicates that the enzyme is pullulanase (Fig. 2) because it hydrolyzes starch to give maltotriose, maltose and glucose whereas amylase after hydrolysis of starch gives limits dextrins, glucose and maltose, and glucoamylases give only glucose (Bertoldo and Antranikian, 2002; Doman-Pytka and Bardowski, 2004).

3.2. Screening of agro-waste

Agro-based industries experience a major problem of waste management. Based on their disposal methods, they are responsible for the pollution of air, water quality, and bad impacts on public health. Chemical methods are generally used for the management of solid waste. Nowadays emphasis has been on biological conversion of plant products. The utilization of organic solid wastes (such as biomass and food waste) for biofuels production is also a promising way to handle the problem of wastes treatment. However, the nutrients are stored in the form of macromolecules (such as cellulose and starch) and should be converted into minor molecular. Han et al. (2017, 2019) developed a novel combined bioprocess based on enzymatic hydrolysis and dark fermentation for hydrogen production (and ethanol production) from food waste (and bakery waste) which could effectively accelerate the hydrolysis speed, improve nutrient conversion efficiency. Furthermore, the techno-economic feasibility should also be evaluated for in-

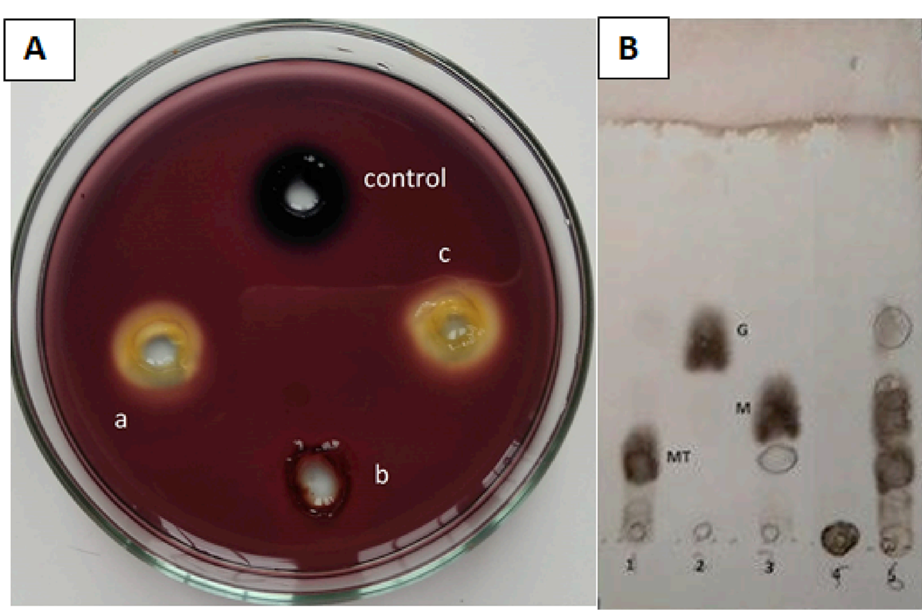

Fig. 2. A- Crude enzyme (50 μl per well) were poured in well and incubated for 12 h and flood with iodine; a, c-clear zone due to hydrolysis of pullulan by the enzyme; control-fermentation media containing pullulan without inoculum; b-fermentation media without pullulan and inoculum; B-TLC profile of hydrolysed products by the enzyme produced by the isolate *Aspergillus* sp. BHU-46, MT-maltotriose; G-glucose; M-maltose; 4, starch without enzyme; 5: starch with enzyme.

dustrial applications (Han et al., 2017). Reports indicate that fungi have the capability of hydrolyzing complex organic compounds for their energy. Agro-industrial wastes such as Wheat bran, rice bran, banana peel, sugarcane bagasse, mosambi peel, orange peel, legume husks, etc. have been used as the substrates for the production of various enzymes (Gawande and Kamat, 1999; Essien et al., 2005; Abdel-Sater and El-Said, 2001). Therefore the Agro-industrial wastes were screened in this study for the optimum production of pullulanase. The wheat bran was found to be the most effective substrate in both SSF and SmF and the most promising isolate *Aspergillus* sp. BHU-46 (Fig. 3). The highest yield for the isolate *Aspergillus* sp. BHU-46 during solid-state fermentation and submerged fermentation was 65.33 ± 2.08U/gds (protein, 5.23 ± 0.46 mg/gds) and 39.33 ± 0.571 U/mL (3.01 ± 0.84 mg/mL) respectively when wheat bran was used as substrate. While in the case of rice bran the maximum yield was 44.66 ± 1.527U/gds (protein, 4.03 ± 0.41 mg/gds) and 41.33 ± 1.154 U/ml (protein, 3.63 ± 0.31 mg/mL). The comparatively lower yield was obtained in sugarcane baggase, orange peel, mausami peel and banana peel by the isolate BHU-46 both in SSF and SmF. As clear from Fig. 3 that the solid-state fermentation gave the comparatively high yield, therefore, SSF was selected for the isolate *Aspergillus* sp. BHU-46.

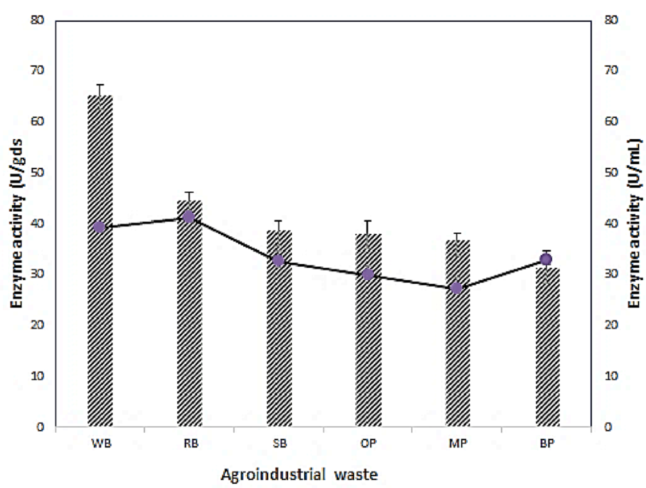

Fig. 3. Effect of different agro industrial substrates and SSF (bar graph) and Smf (line graph) fermentation process on production of pullulanase enzyme; WB-wheat bran; RB- rice bran; SB-sugarcane baggase; OP-orange peel; MP-mosambi peel; BP- banana peel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

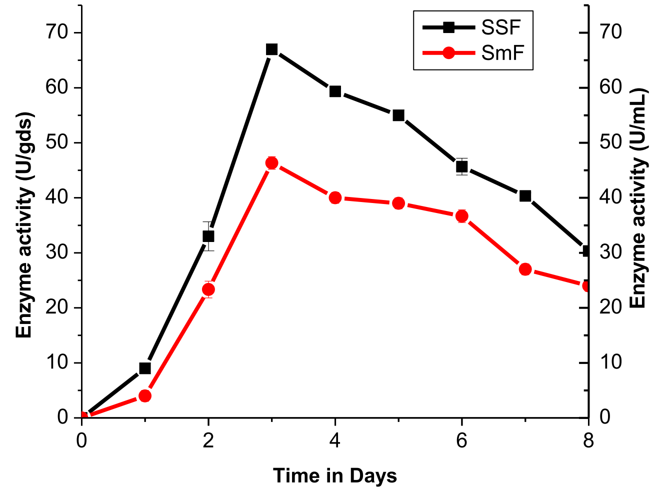

Fig. 4. Time Profile of Pullulanase activity on solid state fermentation and submerged fermentation process.

3.3. Effect of cultivation time

The time course pullulanase production by *Aspergillus* sp. BHU-46 in a medium containing 5 g wheat bran under SSF is displayed in Fig. 4. Maximum Pullulanase activity (67.00 ± 01.00 U/gds) was reached on 3rd day; thereafter the enzyme activity declined. The same pattern was observed in the case of submerged fermentation but the yield was comparatively low (46.33 ± 1.15 U/mL). These findings are similar to results of previous studies on fungi in SSF (Sakano et al., 1972) while others reported after 24 h of incubation (Orhan et al., 2014).

3.4. Effect of moisture and temperature

Low moisture content is known to decrease the metabolic and enzymatic activity probably due to reduced solubility of nutrients from the solid substrate, low substrate swelling and higher water tension (Ramesh and Lonsane, 1990). Therefore, to study the effect of moisture level, the substrate was moistened with different volumes of nutrient solution. It was taken into consideration that the concentration of medium ingredients was not changed. Fig. 5a showed the effect of enzyme production in the medium with 50–80% initial moisture in SSF. Maximal activity (98.66 ± 1.15 U/gds) was attained in the medium with 70% initial moisture content. This could be attributed to the faster growth of the organism at higher moisture content and the subsequent early initiation of the enzyme production. Singhania et al.

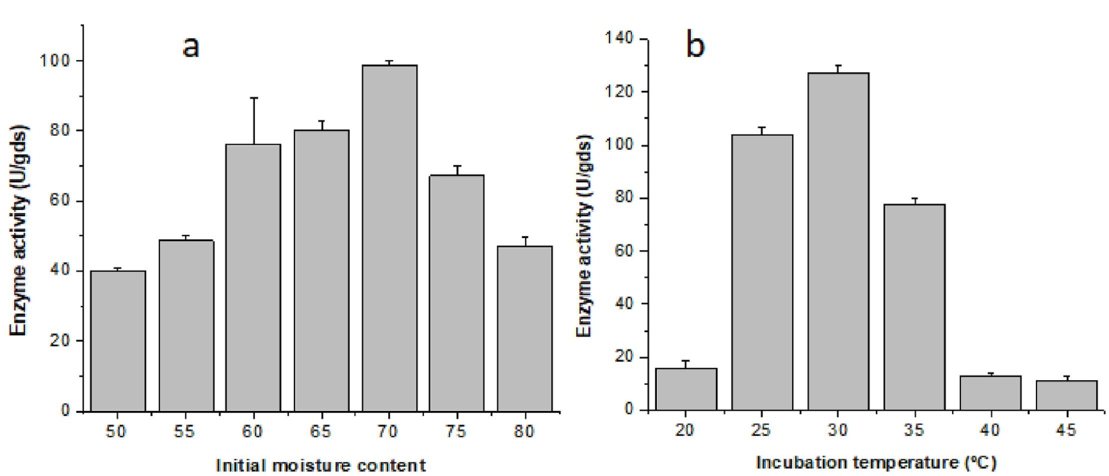

Fig. 5. Effect of initial moisture content (a) and temperature (b) on Pullulanase production by *Aspergillus* sp. BHU-46.

(2007), who found that higher initial moisture content, had a negative effect on cellulase production by *T. reesei* grew on wheat bran under SSF. The maximal cellulase yield was obtained at initial moisture content between 37 and 38%. On the other hand, Farinas et al. (2011) found that the initial substrate moisture content had a positive effect on endoglucanase production by *A. niger* also grown on wheat bran under SSF.

The effect of pullulanase shown in Fig. 5b. The highest production was achieved at 30 °C 127.33 ± 2.51 U/gds and lowest at 45 °C (11.00 ± 1.73U/gds). Orhan et al. (2014) also reported the maximum production of pullulanase fron fungi *Hypocrea jecorina*. While Sakano et al. (1972) reported Pullulan 4-glucanohydrolase, a novel pullulan-hydrolyzing enzyme from Aspergillus niger at 40°C.

3.5. Effect of pH and inoculum size

The effect of pH on the activity is shown in Fig. 6a. The optimum pH for the production of pullulanase was observed at pH 6.0 (69.33 ± 1.15U/gds) followed by 6.5 (68.50 ± 1.00U/gds). The pullulanase activity was also the same over the acidic ranges. However, there was a very slight decrease in pullulanase production was observed above 7 pH, hence this factor was not taken for further optimization using Box-Behnken Design.

Inoculum density is an important consideration for SSF process since overcrowding of spores can inhibit germination and development. In the present study maximal Pullulanase activity was achieved upon using an inoculum of 1 × 10^7 spores/gds (Fig. 6b). Similar observations were obtained by Shamala and Sreekantiah (1986).

3.6. Optimization of physical parameters using Box-Behnken Design

Attempts have been made to optimize three different physical parameters (incubation temperature, and initial substrate moisture and inoculum size) in SSF by using Response surface methodology (Box–Behnken design) to achieve the optimum production of pullulanase by *Aspergillus* sp. BHU-46. The above three physical parameters played a critical role in the production of pullulanase in the wheat bran medium. Box–Behnken design represents the interactive effect of these parameters and to obtain at an optimum level. One variable at a time gave the base points for the selected design. The details of the variables and their variation levels are given in Table S1. The output of the design and results of experiments carried out are given in Table S2. The significant parameters were determined by analysis of variance (ANOVA). ANOVA consists of classifying and cross-classifying statistical results and testing whether the means of a specified classification are significantly different which was carried by Fisher's statistical test. The *F*-value is used to determine whether the test carried out is statistically significant or not. The model equation fitted by regression analysis is given by

$$PA = +394.50 + 5.25X - 6.13Y - 12.63Z - 52.25XY + 26.75XZ - 26.00YZ - 73.50X^2 - 85.25Y^2 - 74.75Z^2$$

where *PA* is the Pullulanase activity (U/g dry substrate), *X the* temperature (°C), *Y is* the moisture content (% (w/w)) and *Z* is the log10 (spores/g dry substrate). The Model *F*-value of 426.07 implied that the model was significant. There was only a 0.01% chance that a "Model *F*-value" this large could occur due to noise. Values of "Probability > *F*″ less than 0.0500 indicated that model terms were significant, however, values greater than 0.1000 indicate the model terms are not significant. In this case, the model terms *X, Y, Z, XY, XZ, YX,* X^2*,* Y^2*,* and Z^2 were found to have significant effect on pullulanase yield. The "Lack of Fit F-value" of 0.52 implies the Lack of Fit is not significant relative to the pure error. There is a 74.03% chance that a "Lack of Fit F-value" this large could occur due to noise. The model determination coefficient *R*2 (0.9990) suggested that the fitted model could explain 99% of the total variation. This implies a good representation of the process by the model. The value of the adjusted determination coefficient (Adj-R^2 = 0.9966) was also very high, indicating a high significance of the model (Table S3). The fitted response surface for the production of Pullulanase by the above model was generated using Design expert and is given in Figs. S2–4. Three-dimensional graphs were produced for pairwise combinations of the three variables, with the third variable in each case settled at its ideal level for pullulanase yield. The graphs are appeared here to highlight the parts played by the different variables additionally to stress the parts played by the physical variables as well as the biosynthetic aspects within the final yield of pullulanase. Generally, circular 3 D plots indicate that the interactions between parameters are negligible. On the other hand, elliptical ones indicate the evidence of the interactions (Muralidhar et al., 2001). Fig. S2 shows the interaction effect of incubation temperature and initial moisture content of the substrate on Pullulanase yield. The response surface graph suggested well-defined optimum variables. There is a significant ($p < 0.01$) positive effect on Pullulalanase yield while increasing the temperature and initial moisture content of the substrate. After the optimum value of temperature (28.6 °C) and moisture (69.9%) further increase in these factors there is a decrease in the production of pullulanase. This is in good agreement with the fact that *Aspergillus* species belonged to mesophilic group. Similarly, above 70% moisture content enzyme activity was decreased which has been supported by the other studies on *Aspergillus* spp. for the production of hydrolytic enzymes (Chimata et al.,

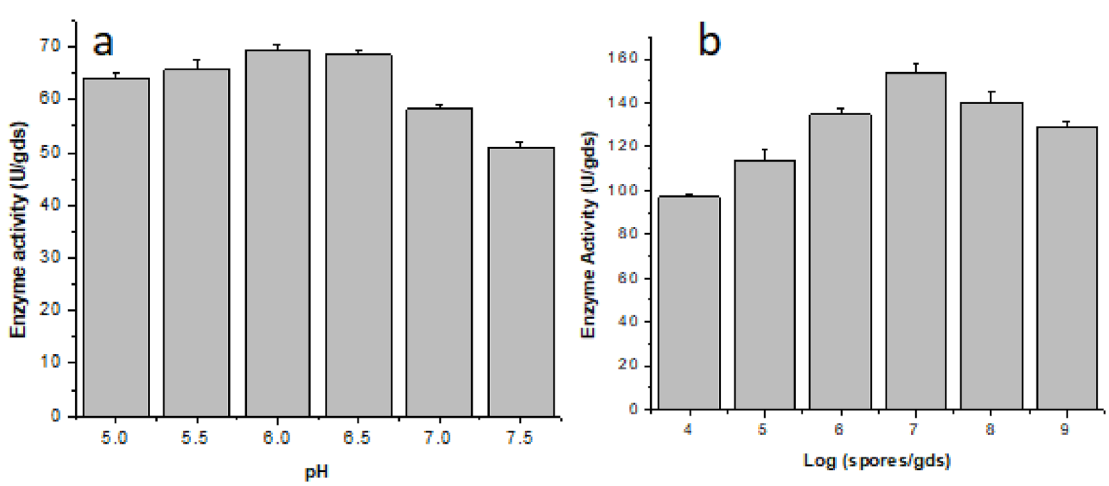

Fig. 6. Effect of pH (a) and inoculum (b) on Pullulanase production by isolate *Aspergillus* sp. BHU46.

2010; Imran et al., 2012). This interaction of the temperature and moisture content was obvious because the temperature is directly related with humidity and water activity, which in turn was a major intrinsic factor governing transport mechanism in microbes (Pandey et al., 2001). In SSF, during fermentation, there is a general increase in the temperature of the fermenting substrate due to respiration (Pandey, 1992; Pandey and Radhakrishnan, 1992). Fig. S3 shows the interaction effect of incubation temperature and inoculum size on production of pullulanase by *Aspergillus* sp. BHU-46 on WBM. There is also a significant ($p < 0.01$) positive interaction between the temperature and inoculum size. After the optimum value (Temperature 28.6 °C; inoculum size, 6.42 log) value, there is decrease in pullulanase production. The shape of the response surface curves showed a high interaction between these tested variables. This interaction is due to the respiratory properties of spores at optimum temperature. At an optimum temperature and minimum inoculum size of spores of fungi highest respiration has been reported (Marakis et al., 1997). Similarly, Fig. S4 Effect of moisture and inoculum size of *Aspergillus* sp. BHU-46 on Pullulanase production. In this case, also there is a significant interaction between these two factors. Lower and higher levels of both the moisture and inoculum size did not result in higher enzyme yields. The shape of the response surface curves showed a significant interaction between these tested variables. It has been reported that in SSF, the quantity of water present in the media is function of the substrate water retention capacity, this quantity should be sufficient for the growth of microorganisms without destructing the solid structure or reduce the porosity of substrate or support (Gervais and Molin, 2003) Moreover, water has a critical impact on physicochemical properties of the substrate, which in turn affect enzyme production (Pandey et al., 1999). Too much water, however, adversely affects oxygen diffusion in the substrate (Kashyap et al., 2003). Moreover, the lower number of cells grows in the production medium at lower inoculum size, due to which the incubation time required to form the desired product increases (Chisti, 2009).

3.7. Validation of the model

To confirm the validity of the statistical experimental strategies and gain a better understanding of pullulanase production, a confirmation experiment with the triplicate set was performed at the specified optimum condition representing the maximum production of pullulanase. Experiments conducted at the optimum condition (temperature 28.62 °C; moisture 69.92%; inoculum size 6.42 log) demonstrated that the Pullulanase yield (396.2 ± 1.33U/gds) was closer to the predicted value (394.5U/gds). The protein concentration of 30.11 ± 0.441 mg/gds was recorded in the final set of experiments. This is an improvement of yield by 6.062 fold increase in yield relative to that obtained from un-optimized physical parameters. The good correlation between predicted and experimental values after optimization justified the validity of the response model and the existence of an optimum point. This showed that the model was useful to predict the pullulanase production as well as optimize the experimental conditions.

4. Conclusion

Pullulanase production can be achieved by using low cost agricultural residual substrates wheat bran. Using this substrate less production time with high enzyme activity of 65.33 U/gds have been achieved. Spore concentration, moisture content of fermentation medium, and temperature was found to have marked effect on the production of Pullulanase from *Aspergillus* sp. BHU-46. By using Box Behnken design there was an improvement of yield by 6.064 fold relative to that obtained from un-optimized physical parameters. As per our knowledge this is first report of pullulanase from *Aspergillus* species produced in solid state fermentation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101423.

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