

Optimization of protease production by *Aspergillus terreus* CJS 127 using *Jatropha* seed cake

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Abstract

The production and optimization of protease using a novel substrate like *Jatropha* seed cake was carried out in the present study. The *Jatropha* seed cake is a byproduct rich in protein generated during biodiesel production. A large quantity of *Jatropha* seed cake (30%) is not being utilized and the disposal of which is a major environmental problem. The optimized conditions for protease production by *Aspergillus terreus* 127a as per the one-factor at a time (O-FAT) method were incubation period-5 days, inoculum size- 1×10^7 spores/ml, pH- 7.0, moisture content 60%, temperature-30°C, which resulted in the protease activity of 5951 U/g. The protease activity was further optimized by using Taguchi's method, and the activity was increased by two folds (10,283 U/g) when compared to O-FAT method. The optimum parameters for maximum protease production under Taguchi's method were four days of incubation, inoculum size 10^7 spores/ml, pH 7.0, 50% moisture content and 25°C incubation temperature. Our results suggested that solid state fermentation process is a tool to utilize the low-cost substrate like *Jatropha* seed cake for the production of industrially important enzyme such as protease.

Keywords Protease • *Aspergillus terreus* CJS-127a • *Jatropha* seed cake • O-FAT • Taguchi's method

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Introduction

Jatropha curcas (Euphorbiaceae) occurs naturally in Central America but has been cultivated in many tropical and subtropical areas of America, Africa and Asia (Heller J, 1996). *J. curcas* seed contains high amount of oil that can be converted into biodiesel upon transesterification. Apart from the oil, the seed cake or kernel meal leftover has gained enormous interest for their utilization (Makkar et al., 1997). *J. curcas* has been identified as potential biodiesel crop as an alternative fuel is accorded high priority by government of India and National Biofuel Programme (Khare DK, 2006). *Jatropha* seeds found to contain 60-70% oil and the seed cake generated after biodiesel extraction is rich in protein (>30%). The seed cake also has several anti-nutrients and toxins like phorbol esters which make it not suitable for use as feed ingredient (Sharath et al., 2014). A large quantity of seed cake is generated after biodiesel extraction and the disposal of the seed cake is an environmental issue which needs to be addressed.

The deoiled *Jatropha* seed cake (JSC) can serve as a rich source of nitrogen and carbon source for growth of microorganisms and enzyme production. The seed cake also has several of both essential and non-essential amino acids present in the kernel meal (Makkar et al., 2008). Solid-state fermentation has been a viable technique for utilization of agro-industrial by-products and biomass. The nutrients present in the substrate support microbial growth secreting useful enzymes while growing on solid substrate fermentation. The presence of the high amount of protein in JSC, therefore, could stimulate protease production in microorganisms in solid state fermentation. The protease enzyme accounted for 60% of total production of industrial enzymes (Rao et al., 1998). Proteases have application in a diverse range of industries, such as food, medicine, detergent and leather. The fungal proteases offer a distinct advantage over bacterial enzymes in terms of ease of downstream processing (Laxman et al., 2005). Proteases are reported to be produced using various agro industrial wastes (Joel et al., 2011 and Nehra et al., 2002), but there are only few studies on the utilization of JSC for the production of enzymes by SSF (Mahanta et al., 2008; Joshi et al., 2011; Thanapimmetha et al., 2012 and Mohankumar et al, 2014).

The enzyme production by any microorganism is dependent on various physicochemical parameters. The optimization of various physicochemical characters is essential to get a better yield of enzyme production. These parameters should be optimized in such a way that yield must be maximum without compromising cost and stability of the system. Optimization of media

components by the traditional 'one-variable-at-a-time' strategy involving changing one independent variable is the most frequently used operation in biotechnology (Haaland PD, 1989). This strategy is extremely time consuming and expensive when a large number of variables are considered and incapable of detecting the true optimum due to the interactions among the factors. In Taguchi's method, variables or factors are arranged in an orthogonal array (OA). The orthogonal array properties are such that between each pair of columns each combination of levels (or variables) appears an equal number of times. Due to an orthogonal layout, the effects of the other factors can be balanced and give a relative value representing the effects of a level compared with the other levels of a given factor. In orthogonal array experiments, the number of test runs is minimized, while keeping the pair-wise balancing property (Roy RK, 1990).

The objective of this present investigation was production and optimization of protease enzyme from *Aspergillus terreus* CJS-127a using JSC by Taguchi's method. For the optimization of protease production, parameters like incubation time, inoculum size, pH, moisture and temperature were selected, and their levels were selected with the help of O-FAT (one factor at a time) method initially. Taguchi's method was designed with five factors at two levels with layout of L16. The present study was focused to determine optimum condition for protease production by *A. terreus* CJS-127a using Taguchi's method.

Material and methods

Microorganism and solid state fermentation

The organism used in the study was a locally isolated fungi namely *A. terreus* CJS-127a. The culture was maintained on Czapek Dox agar media and subcultured once in 15 days. *Jatropha* seeds were obtained from the local market, and seed cake was prepared using the hydraulic press after extraction of oil. Solid-state fermentation (SSF) was carried out using 5 gm of *J. curcas* seed cake powder in 150 ml capacity conical flask. 1.5ml of distilled water was added to the flask and was autoclaved for 20 min at 121°C. 2 ml of sterile physiological saline was added to 7 days old fungal slant cultures of *A. terreus* CJS-127a. The spores were gently scrapped on to the saline. Spore count was done with the help of haemocytometer. 0.5 ml of spore suspension with 10^7 spores was added to each flask containing JSC. All the flasks were incubated for the respective number of days at 30°C.

Extraction of enzyme

After the growth of the organism by SSF, 25 ml chilled distilled water was added to each flask. Flasks were kept on an incubator shaker at 150 rpm for 30 min. The culture fluid was filtered using a filter paper. The above procedure was repeated by adding 25 ml of distilled water again to the residue. The filtrate was pooled and centrifuged at 11740 g for 10 min at 4°C. The supernatant was used as the source of enzyme and stored at 4°C until use.

Enzyme assay

Protease activity was done as described by Mohan et al, (2014). 3 ml of casein (Hammersten-0.6%), (SRL chemicals, India) dissolved in 0.05 mol l⁻¹ of sodium phosphate buffer pH 7.0 was used as a substrate. Suitably diluted enzyme of 0.5 ml was used for the assay and incubated for 20 min at 37°C. The reaction was stopped by addition of 3.0 ml TCA (110 mM) and incubated for 30 min. The supernatant was recovered by filtration using Whatman filter paper No.1, and 2.0 ml of filtrate was taken. 5.0 ml of sodium carbonate (500 mM) and 1.0 ml of diluted Folin's reagent (Folin's reagent:distilled water 1:1) were added and incubated for 30 min. The solution was filtered after the addition of reagents and was read at 660 nm. Tyrosine liberated while casein hydrolysis was measured in the supernatant using the method of Lowry et al, (1951). 1951. One unit of protease was defined as the amount of enzyme required to release 1 µg of tyrosine/ml/min under assay conditions. The protease activity was expressed as Units/gram (U/g) of the substrate used in SSF.

Optimization of media conditions by O-FAT method

For selecting levels of various parameters for optimization of enzyme production, O-FAT method was used. The parameters selected were incubation period (1-6 days); inoculum size 10²-10⁸ spores/ml; pH 5.0-9.0; moisture content 30-70% and temperature 25-40°C. Initially, the experiment was carried out keeping the following parameters constant that is 5 days of the incubation period, 1x10⁷ spores/ml as inoculum, pH 7.0 with 40% moisture and incubation temperature at 30°C.

Taguchi's method

Minitab 15 Taguchi software (e-academy) was used to optimize the protease production with the help of Taguchi's method. As per O-FAT results for optimization of different parameters, experimental design of 5 factors were selected at 2 levels (range) for further studies which are as follows: incubation period - 4 and 5 days; inoculum size- 10⁶ and 10⁷ spores/ml; pH- 6.0 and

7.0; moisture - 50 and 60%; temperature- 25 and 30°C. SSF experiments aimed for the protease production by *A. terreus* CJS-127a employing the layout of L-16 (2^5) orthogonal array system. In this study, all the graphs were represented in terms of protease activity (U/g) as a value of the factors (Table 1). All experiments were done in duplicates and average values were given.

Table 1: Taguchi's experimental design matrix and production of protease enzyme by *A. terreus* CJS 127a

| Exp. no. | Incub- ation time (days) | Inoculum size (spores/ml) | Moisture | | Temper -ature (°C) | Observed Protease activity. (U/g)±S.E. | Predicted Protease activity | S/N Ratio |
|----------|-----------------------------------|---------------------------------|----------|-----|--------------------------|---|-----------------------------------|--------------|
| | | | pH | (%) | | | | |
| 1 | 4 | 10 ⁶ | 6.0 | 50 | 25 | 8333.20±0.67 | 8300 | 98.42 |
| 2 | 4 | 10 ⁶ | 6.0 | 60 | 30 | 7601.95±0.53 | 7590 | 97.62 |
| 3 | 4 | 10 ⁶ | 7.0 | 50 | 30 | 7744.14±0.28 | 7732 | 97.78 |
| 4 | 4 | 10 ⁶ | 7.0 | 60 | 25 | 9338.67±0.83 | 9358 | 99.41 |
| 5 | 4 | 10 ⁷ | 6.0 | 50 | 30 | 7617.18±0.37 | 7610 | 97.64 |
| 6 | 4 | 10 ⁷ | 6.0 | 60 | 25 | 7810.15±0.74 | 7815 | 97.85 |
| 7 | 4 | 10 ⁷ | 7.0 | 50 | 25 | 10283.20±0.63 | 10135 | 100.24 |
| 8 | 4 | 10 ⁷ | 7.0 | 60 | 30 | 8729.29±0.86 | 8839 | 98.82 |
| 9 | 5 | 10 ⁶ | 6.0 | 50 | 30 | 3545.72±0.82 | 3628 | 90.99 |
| 10 | 5 | 10 ⁶ | 6.0 | 60 | 25 | 5281.25±0.79 | 5172 | 94.45 |
| 11 | 5 | 10 ⁶ | 7.0 | 50 | 25 | 6738.67±0.53 | 6530 | 96.57 |
| 12 | 5 | 10 ⁶ | 7.0 | 60 | 30 | 4930.85±0.85 | 4924 | 93.86 |
| 13 | 5 | 10 ⁷ | 6.0 | 50 | 25 | 7434.37±0.82 | 7438 | 97.42 |
| 14 | 5 | 10 ⁷ | 6.0 | 60 | 30 | 6190.23±0.42 | 6159 | 95.83 |
| 15 | 5 | 10 ⁷ | 7.0 | 50 | 30 | 8419.53±0.45 | 8411 | 98.51 |
| 16 | 5 | 10 ⁷ | 7.0 | 60 | 25 | 7467.08±0.62 | 7587 | 97.46 |

Results and discussion

The production of protease was optimized by O-FAT method by varying different parameters. As per the O-FAT results the protease activity was maximum on 5th day of incubation with

inoculum size of 10^7 spores/ml, pH-7.0, moisture content at 60%, temperature at 30°C. The maximum protease activity under these conditions was found to be 5,951U/g (Fig. 1^{a-d}).

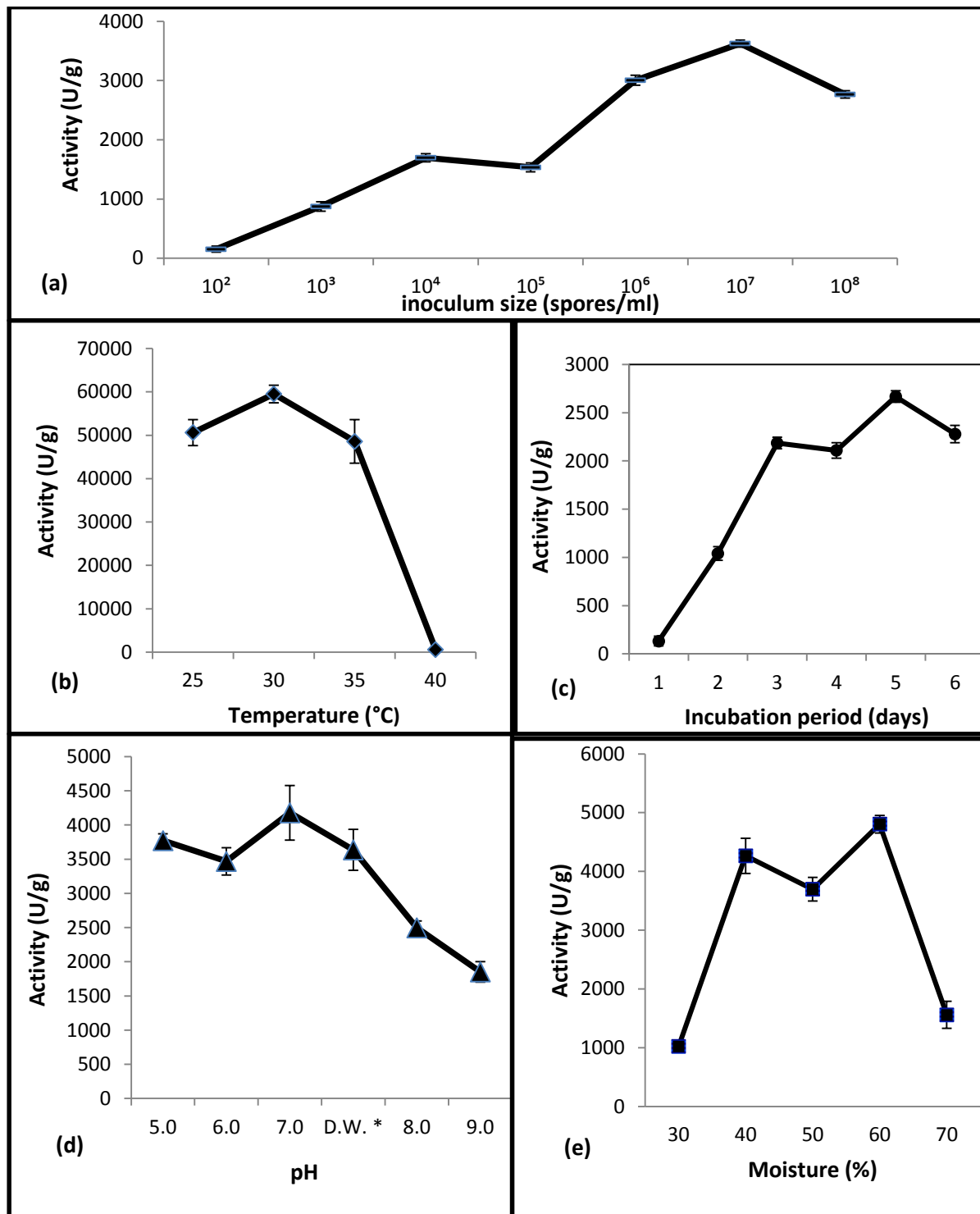


Figure 1: Optimization of parameters for protease production by *Aspergillus terreus*

CJS- 127a by O-FAT method

Optimization of protease production by Taguchi's method

Taguchi's method was used to get process information to know optimal conditions of physicochemical parameters for a particular process using a minimum number of experiments. The properties of an orthogonal array are such that, between each pair of columns each combination of levels appear an equal number of times. Due to the orthogonality of the layout, the effects of the other factors will be balanced and give a relative value representing the effects of a level compared with the other levels of a given factor. The symbolic designation of these arrays indicates the main information on the size of the experimentation which has 16 trials. In this study, two levels are designed with different factors as shown in Table 1. The production of protease was highly dependent on the cultural conditions. An L16 orthogonal experimental design was used to investigate five different culture components such as, pH, incubation time, inoculum size, moisture content, temperature. The experiments were conducted using two levels for each factor. Statistical analysis of the data pointed out that the optimal levels of the different factors for protease production were incubation time 4 days, inoculums size 10^7 , pH 7.0, moisture content 50%, temperature 25°C (Trial 7). Under these conditions, the maximum protease activity was 10,283 U/g. The average effect of the factors at the assigned levels on the protease production by *A. terreus* CJS 127a is shown in Table 2. This table shows the influence of five individual factors on the protease yields. At optimized conditions for enzyme production largest value for S/N ratio was observed. Hence under these conditions, system was highly stable to the noise. Using O-FAT method, Thanapimmetha et al, (2012) and Mohan et al, (2014) have shown that the protease activity was maximum on 4th day of incubation, i.e., 3094 and 3366 U/g respectively. Similarly, Mahanta et al. have reported that the maximum protease activity of 1818 U/g at 72 h by *Pseudomonas aeruginosa* (Mahanta et al., 2008). The highest protease activity on rice bran was about 1400 U/g compared with the activity of about 1000 U/g obtained on wheat bran at 86 h using *A. oryzae* (Chutmanop et al. 2008). The results of our study showed that the organism could grow on JSC and produce protease activity much higher than the proteases reported using conventional SSF substrates like rice bran and wheat bran.

Table 2: ANOVA for protease production by *A. terreus* CJS-127a

| Source | DF | F value | p-value |
|---------------------------|----|---------|---------|
| Incubation time | 1 | 31.80 | 0.005 |
| Inoculum | 1 | 11.38 | 0.028 |
| pH | 1 | 10.11 | 0.034 |
| Moisture | 1 | 0.80 | 0.422 |
| Temperature | 1 | 6.53 | 0.063 |
| Incubation x Inoculum | 1 | 6.02 | 0.070 |
| Incubation x pH | 1 | 0.01 | 0.910 |
| Incubation x Temperature. | 1 | 0.01 | 0.942 |
| Inoculum x pH | 1 | 0.36 | 0.581 |
| Inoculum x Temperature. | 1 | 1.53 | 0.283 |
| pH x Temperature | 1 | 0.00 | 0.976 |
| Residual Error | 4 | | |
| Total | 15 | | |

Mahanta et al. have reported maximum protease production of 1818 U/g on 3rd day using *P. aeruginosa* (Mahanta et al., 2008). The moisture content was found to be optimum at 50% and substrate pH at 6.0. Based on the literature reports, the moisture content influenced the protease production in microorganisms (Chutmanop et al., 2008). The effect of an increase in moisture content was a decrease in porosity of the substrate, leading to the reduction of gas exchange. Whereas low moisture content had an influence on the sub-optimal growth of microbes (Pandey et al., 1999). Mohan et al, (2014) have reported that protease activity was 3366 U/g at 96 h with moisture content of 50%. Thanapimmetha et al, (2012) have reported that the optimum conditions for the protease production by *A. oryzae* obtained from the experiment were 45% moisture content of the substrate, 10% inoculums size, 30°C incubation temperature when deoiled *J. curcas* seed cake mixed with cassava bagasse ratio 4:1 was used as a substrate at 84 h of incubation time. In the case of *A. oryzae* grown on wheat bran and rice bran (Chutmanop et

al., 2008), the protease activity was very low compared to the activity reported by using JSC. High protease activity in *A. terreus* CJS-127a was due to its ability to utilize protein present during SSF. JSC has the highest protein content of (> 30%) when compare to the protein content in rice bran and wheat bran which were 13-14% and 12-17% respectively (Chutmanop et al., 2008). The optimum inoculum size for protease production by *A. terreus* CJS-127a was 10^7 spores. The size of the inoculum has significant influence on the microbial synthesis of the enzyme. Typically, an increasing in inoculum size advanced the microbial growth and enzyme production. But too high inoculums could lead to the nutrient competition of fungal on limited substrate (Yalemtesfa et al., 2010). The optimum incubation temperature on the enzyme production was dependent on the strain of the microbes. The optimum temperature for protease production by *A. terreus* CJS-127a was 25°C. Mohan et al, (2014) have reported that the maximum protease production by *A.versicolor* CJS-98 was at 25°C. Since a certain amount of heat was generated during SSF, an incubation temperature must be directly proportional to the metabolic activities of the microbes (Pandey et al., 2003). The protease production decreased when incubation temperature was raised to 30°C. The optimum conditions for the protease production by *A. oryzae* obtained were 45% moisture content of the substrate, 10% inoculum size, 30°C incubation temperature, when deoiled JSC mixed with cassava bagasse ratio 4:1 as porous substrate at 84 h of incubation time (Thanapimmetha et al., 2012). The analysis of the data was done by using the Minitab program. Mean response of protease activity against each parameter was plotted at two levels by Minitab program (Fig 2). If there was a small deviation between the levels of the incubation period, inoculum size, pH and temperature that could lead to a greater change in mean response of protease activity or the angle between equivalence line and the plot was more. The protease activity was more influenced by incubation period, inoculum size, pH and temperature than moisture because, in the case of moisture, the angle between the line of equivalence and the plot was lesser. Minitab was plotted to know the response of means for each factor level (Fig 2.). The protease production almost doubled at 10,283 U/g by Taguchi's method when compare to the O-FAT method. The incubation period was also reduced from 5 days to 4 days by Taguchi's method. The optimum levels in each factor were 50% moisture content, 10^7 spores inoculums size, 25°C incubation temperature and 96 h incubation time. A quantitative measure of the influence of individual factors was obtained from the analysis of variance (ANOVA) which is shown in Table 2. The main objective of ANOVA was to know

from the resulting level of variations each factor causes relative to the total variation observed in the result. From the results of ANOVA in Table 2, the incubation and inoculum concentration factors had the largest variance for protease production. The variance within the factors and between the factors was analyzed.

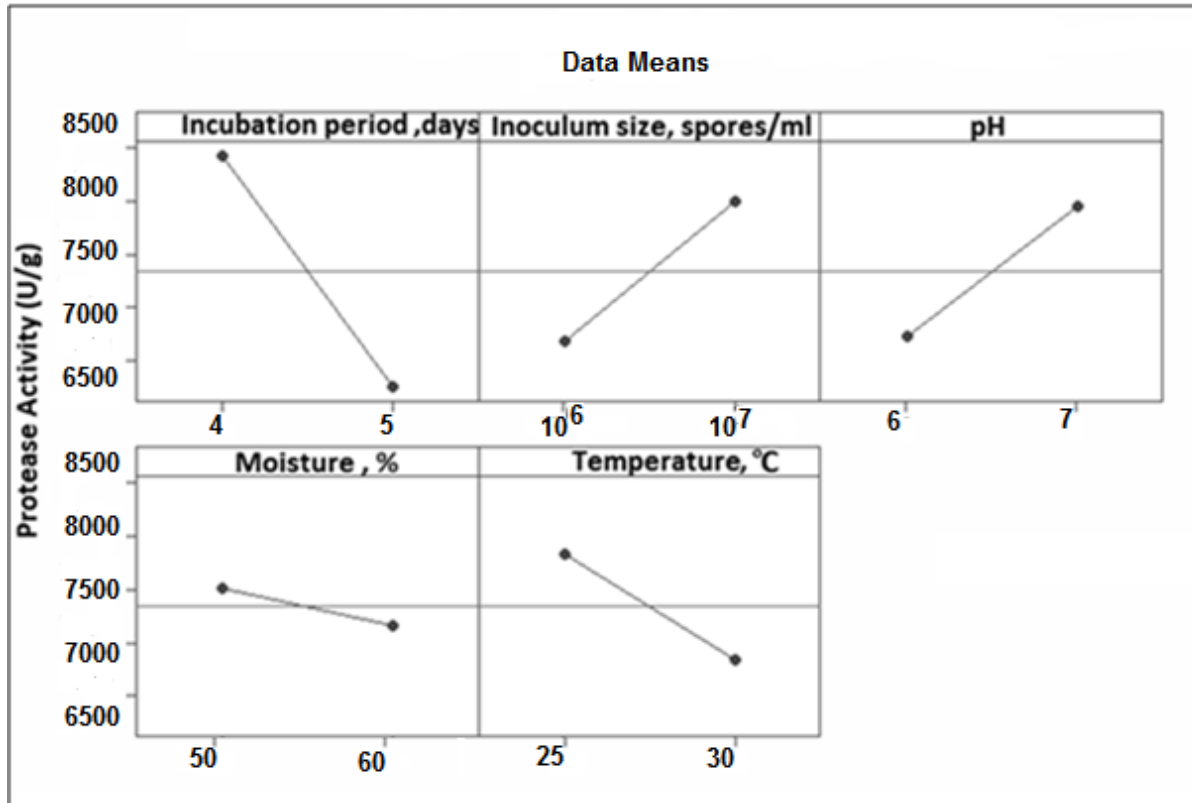


Figure 2: Graph showing the effect of each factor contribution in the protease enzyme production by *Aspergillus terreus* CJS- 127a.

There was a significant association between incubation time, inoculum and pH as per their p values are less than 0.05. Null hypothesis was not true for incubation time; inoculum size, pH, temperature and interaction between incubation time and inoculum size as their F value was greater than F (a-level=3.35) value. The incubation time, inoculum size, pH, temperature and interaction between incubation time and inoculum size have p value lesser than 0.05 and F value greater than 3.35; hence, these are significant parameters to predict the protease activity. This statistical model for the enzyme production was 95% significant.

The present study demonstrated the protease activity by *A. terreus* CJS-127a using JSC as substrate for enzyme production was, therefore, a good strategy for feasibility of utilization of the JSC. The main purpose of this study was for utilization of JSC for protease enzyme production and optimization of process parameters by *A. terreus* CJS-127a using Taguchi's orthogonal array. The Taguchi's method reduced production time and important parameters affecting protease production were identified. Thus, manufacturing and operations costs can also be greatly reduced. Taguchi experimental design involves the study of given system by a set of independent variables (factors) over a specific region of interest (levels) by identifying the influence of individual factors, establishing the relationship among variables and also the performance at the optimum levels (Sreenivas et al., 2008). By studying the main effects of each of the factors, the general trends of the influence of the factors towards the process can be predicted and controlled such that a lower or higher value in a particular influencing factor produces the preferred result. The levels of factors, to produce the best results can be predicted. Optimal conditions for protease production by *A. terreus* CJS 127a were evaluated by choosing one factor at a time approach and also by Taguchi's method. By using Taguchi's method, the protease activity was increased two folds viz., 10,283 U/g. Thus the solid state fermentation of *Jatropha* seed cake is an environmental friendly process for the production of protease.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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