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Optimization and Production of Alkaline protease from *Bacillus subtilis* IAS01 using agro-industrial by-product under SSF

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Abstract

Edible and non-edible oil cake utilized for alkaline protease production by Bacillus subtilis IAS01 (KF 761633) in SSF. Experimentally revealed that groundnut oil cake and palm oil cake mixed in a ratio of 5:5 (w/w) supported for protease production when compared to individual and mixed substrate. In addition to attain the maximum yield of this enzyme in appropriate fermentation conditions viz. moisture 30%, 72 h incubation, 20% inoculum, pH 9.0 and temperature 37°C with recorded 835.13±5.04 *units per gram of substrate. These results indicate that mutual inoculants of groundnut oil cake and palm oil cake are potential substrate for alkaline protease production at low quantities of substrate. In feature its best opportunity for protease production at industrial level.*

Keywords: Agro industrial by-products, alkaline protease, *Bacillus subtilis* IAS01, culture conditions, one-way ANOVA.

Introduction

Deals with utilization of cheapest available material in agricultural product or by product and are subjected to the study. Previously reported edible and non-edible oil cakes have enormous amount of fiber containing nutrient comparing to crop residue, less fibrous, more concentrated and has a higher nutrient content[1]. In general, microbial Proteases are occupies the important position in industrial enzymes usages, when compared to other enzyme like amylase, lipase, lactase, amyloglucosidase and cellulose. They also produced by a broad variety of living things such as bacteria, fungi, yeasts, moulds and are also found in plants and animal tissues. Among them microbial protease are the mainly acceptable enzyme for 60-65% of marketed throughout the world[2,3]. Nowadays, in modern countries, the use of particularly alkaline proteases in a variety of industrial processes like textile, food, pharmaceuticals, chemicals, tanneries and waste water treatment[4]. Currently, a large proportion of commercially available alkaline proteases are obtained from *Bacillus* strains by solid state fermentation[5]. Recently SSF has generated much interest, because of lower manufacturing costs by utilizing untreated or moderately processed raw materials us well as agro-industrial by product. Nowadays, SSF has shown a significant prospective in the industrial production of a secondary metabolites and extra cellular enzyme such as protease, amylase, lipase and *etc*[6,7]. This mode of study deals with the nearby available nutrient rich content of various agro-industrial by products related to (edible and non-edible) the value addition of oil cakes is the residue obtained after oil extraction from greater part of oil seed such as sunflower oil cake (SuOC), sesame oil cake (SOC), coconut oil cake (COC), groundnut oil cake (GOC), palm kernel oil cake (PKC) and castor oil cake are distinguished raw material from which they com and also rich in protein and most are valuable food for microbial growth and its alternative way of farm animals.

India is one of the leading producers of oil cakes in the world and occupies vital position in Indian agricultural economics survey, is an easily available cheap product which finds use only as an animal feed and fuel in rural areas. Oil cake, are especially groundnut oil cake, being rich sources of carbon, nitrogen and also present essential amino acid, can be serving as a suitable substrate for the growth of microorganisms [8, 9]. However, with decreasing in a production cost of industrial processes and value addition to agro-industrial by product, Oil cakes are rich in fibers, proteins and multi benefits when used as substrates in rising bioprocesses for the production of industrial enzyme and organic acid. Edible and non edible oil crop is versatile and economical group of oil in many countries of all over the world. Importance of this study is to inspect the locally available low cost agro-industrial substrate for alkaline protease production in SSF by using *Bacillus subtilis* IAS01 (KF: 761633) and optimize its culture condition. In this communication, we have shown for the first time the microbial alkaline protease production by using mutual inoculation of groundnut oil cake and palm oil cake as fermentation substrate in this study. In addition suggested that way to increase the edible and non edible crop cultivation for improve the green environment of the world.

Material and Methods

Chemicals and Microorganism: Analytical reagent and chemicals were purchased from national scientific branch at puducherry, Union Pradesh, India. The bacterium *Bacillus subtilis* IAS01 (KF 761633) used in this study it was previously isolated from coastal regions of rhizosphere soil in cuddalore

District, Tamil Nadu. It was maintained with aseptically in nutrient agar slant and keep in refrigerator for further research.

Preparation of inoculums: A volume of 100 ml nutrient broth was taken in a 250-ml conical flask and inoculated with a loop full of pure culture from 24 h old plate and kept at 30°C in a shaking incubator. An after 24 h of incubation, 1 ml of this nutrient broth culture was used as the inoculums containing 3×10^6 CFU/ml of viable colonies.

Screening and production of enzyme through SSF: Different kinds of agro industrial by product such as edible oil cake *viz.* coconut oil cake (COC), groundnut oil cake (GOC), sesame oil cake (SOC) and non edible oil cake *viz.* castor oil cake, sunflower oil cake (SuOC) and palm oil cake (POC) were obtained from local market and were washed with plenty of tap water followed by with distilled water to remove unwanted or irrigated particles from an oil cake surface and after washed them in hot water (75-80°C) for 20 min followed by oven drying at 45°C for the purpose to avoid microbial contamination during the fermentation. Further 10g of each substrate was weighed into a 250-mL conical flask and moistened at 20 % (w/v) distilled water adjust pH with using Tris–HCl buffer (20 mM, pH 7.0). The flasks were autoclaved for 20 min at 121°C. After cooling, 1 ml of culture was added in to the contents and thoroughly mixed the flasks were incubated at 30°C for 48 h.

Protease extraction and assay: To withdraw 10g of fermented substrate from after reached corresponding fermentation medium then add to 20-30 ml of same buffer and to keep on a rotary shaker at 150 rpm for 1 h. The extract was primarily filtered with bacteriological filter paper further filter with small size of 45µ filter paper by using vacuum pump and extracts were collected up to 100 ml, centrifuged at 12,000×g, at 4°C for 15 min, and the supernatant was used as enzyme source for protease assay and 70% ammonium sulfate precipitate was subjected throughout protease characterization test. After that corresponding protease activity was quantitatively analyzed by the using earliest method described by Esakkiraj P.[10] and the protein content in the samples was estimated by Lowry O.H.[11]. The amount of protein present in the sample was calculated from the standard curve.

Optimization of process parameters: Interestingly, the physical parameters can gradually stabilize the microbial growth and fertility. Experimentally were subjected in a sequential order, moisture contents of the substrate in % (10, 20, 30, 40, 50, 60, and 70) maintained before autoclaving (distilled water were adjusted with 0.05M Tris-HCl buffer for pH 7.0). The protease activity was determined for every 12 h of fermentation up to 84 h incubation. In order to determine the effect of inoculum percentage on protease production were optimized (10-70%), evaluate the acidic to alkaline range of pH (4-13) and temperature (30-55°C) were tested for their effect on protease production.

Statistical analysis: Statistical analysis was performed using IMB SPSS (20) version software. One way analysis of variance (ANOVA) followed by Duncan multiple range test method was used to correlate the difference between individual variables and its data was pointed out significant $p\leq 0.05$.

Results and Discussion

Screening of edible and non edible oil cakes as substrate for protease production through SSF: SSF is an economically viable, practically acceptable technology for the large-scale bioconversion and biodegradation processes. During the study different (edible and non edible oil cakes) solid substrates *viz.* coconut, groundnut, sesame, sunflower, palm and castor oil cake were screened individually and mixed conditions for alkaline protease production by using strain 'IAS01' under SSF. An after experimental assay it was concluded that the mixture of groundnut oil cake and palm oil cake 5:5(w/w) were proved to be an efficient combination for alkaline protease production. The result presented in table-1 showed that highest enzyme activity (542.31±5.08U/g), when compared to individual and mixed substrate followed by the fermentation condition pH 7.0, temperature 30°C, incubation 48 h, respectively both groundnut oil cake and palm oil cake are used as the substrate followed by culture optimization study due to improve the yield of protease production. Similarly Joo H.S.[12] reported oil cakes are preferably stabilize the nutrient for bacterial growth under SSF condition with rendering both carbon, nitrogen sources to be reported good substrate for enzyme production using *Bacillus* sp. The present study revealed that capability of edible and non-edible oil cake being rich sources of carbon, nitrogen and some essential micro and macro nutrient can thus serve as a suitable substrate/support for the growth of corresponding strain IAS01. Similarly Uyar F.[13] who reported that wheat bran has been the preferred choice for microbial protease production through solid state fermentation. Present study documented to the protease production from *Bacillus subtilis* IAS01 on a mixture of groundnut and palm oil cake (5:5 w/w) is much better than protease production on other tested substrates.

Effect of initial moisture content and incubation period on protease production: The maximum protease productions were observed in the range of 20-40% moisture and the highest enzyme production was found in 30% result showed in table 2. Similarly Uyar[13] who reported based on the result trough *Bacillu*s sp produce alkaline protease in the same condition under SSF. Similarly Chellappan[14] observed that, the higher moisture content may cause decreased porosity, loss of particle structure, development of stickiness and reduction in gas volume and moisture level as a most important parameter for predicting microbial fertility. A reduction of enzyme production at high and low moisture content due to decrease porosity of the substrate and also this may be due to the difference in nature of a solid substrate used for fermentation. Therefore, in solid state fermentation the intensity of microbial growth generally depends on the initial moisture level and it indirectly affects the production.

Table-1
Effect of substrate on alkaline protease production from IAS01 under SSF

Substrate	Weight (g)	Total protein (mg/ml)	Protease activity (U/g)
Edible oil cake			
Coconut oil cake	10	1.97±0.08	237.26±5.06[g]
Groundnut oil cake	10	2.24±0.12	274.36±4.09[f]
Gingili oil cake	10	1.72±0.05	190.33±2.07[h]
Non edible oil cake			
Palm oil cake	10	1.40±0.09	153.83±2.07[i]
Sunflower oil cake	10	1.05±0.05	145.00±3.20[j]
Castor oil cake	10	1.54±0.20	179.70±3.10[a]
Mixed form Groundnut oil cake + sesame oil cake	5+5	2.03±0.32	318.26±3.06[e]
Groundnut oil cake + coconut oil cake	5+5	3.01±0.10	501.80±6.04[c]
Groundnut oil cake + castor oil cake	5+5	2.39±0.35	451.26±3.13[d]
Groundnut oil cake + sunflower oil cake	5+5	2.57±0.17	516.29±6.02[b]
Groundnut oil cake + palm oil cake	5+5	3.05±0.12	542.31±5.08[a]

Screening of different agro-industrial by-product for alkaline protease production by IAS01 under solid state fermentation, every one values of given the column are statistically indicated six replication for analyzed by one way ANOVA followed by DMRT of IMB SPSS (20) version software.

The effect of incubation time on protease production was studied over a period of 12-84 h at 30°C. Result of this study showed that the protease production increased in the range of period (48-84 h), the highest enzyme production was recorded in the period of 72 h (table 3). These results are in accordance with similar observations made by Okafor U.O.G.[15].

Table 2
Effect of moisture content on alkaline protease production from IAS01 under SSF

Moisture (%)	Total protein (mg/ml)	Protease activity (U/gds)
10	2.04±0.21	457.08±6.90
20	2.82±0.20	504.81±9.67
30	2.12±0.15	603.08±12.65
40	2.04±0.21	453.26±7.36
50	1.85±0.12	433.26±7.36
60	1.69±0.19	425.99±5.59
70	1.10±0.09	373.84±8.25

Values were given as mean ± SD of six experiments in each moisture percent (ANOVA followed by DMRT) significantly at p≤0.05. Under fermentation set *viz.* initial pH 7.0, temperature 30 °C, 10% inoculum and incubated at 48 h.

Table-3
Effect of incubation period on protease production from IAS01 under SSF

Incubation period	Total protein (mg/ml)	Protease activity (U/gds)
12	1.33 ±0.11	224.02±7.31
24	2.09±0.13	282.79±5.54
36	2.13±0.13	318.29±5.86
48	2.17±0.10	453.26±7.78
60	2.49±0.09	617.12±7.62
72	2.94±0.20	641.59±3.68
84	2.03 ±0.06	572.84±6.20

Values were given as mean ± SD of six experiments in each period (ANOVA followed by DMRT) significantly at p≤0.05. Under the culture conditions *viz.* 30% moisture at initial pH 7.0, temperature 30 °C and 10% inoculum.

Effect of inoculum size on protease production: In order to establish the effect of inoculums size of protease production from *Bacillus subtilis* IAS01, the culture was inoculated with various levels inoculums (10%, 20%, 30%, 40%, 50% and 60%) were used to study. The highest enzyme activity was obtained at 30% of inoculum this variance indicated in (table 4). However, the low level of inoculum present low level of cell for the result to take long duration, however, increase with the inoculum level beyond 40% (v/w) when adversely affected the enzyme production due to exhaustion of nutrients in the fermentation mash, whereas higher concentration may produce excessive biomass and deplete substrate for the nutrients necessary for product. Similarly Shafee N.[16] who reported decrease in protease production at inoculum size below the optimum may be due to extended lag phase that result in insufficient number of microorganisms to ferment the solid substrate. Another observation by Kumar R.[17], reported that when increase inoculum sizes the growing microbial cells may have created stressful conditions such as depletion of nutrients, pH fluctuation change in availability of oxygen, and competition to access limited resources to result low protease production. Therefore in this study that implies importance of controlling inoculum size in yielding high protease.

Effect of pH and Temperature on protease production in SSF: The effect of varying initial pH values on protease production is shown in (table-5). Hence, at higher and lower pH of the bacterium may be slow growth and thus enzyme production was also inhibited because it dramatically affects the

microbial growth. Similarly Hadeer L.[18] who reported that alkaline protease production from *Streptomyces* sp. CN902 under SSF at pH 9.0. Therefore, in subsequent experiments the pH of the fermentation medium was kept at pH 9.0. At a higher and lower pH, the metabolic action of the bacterium could have been suppressed and decreasing the enzyme production. In initial temperature of the fermentation condition was showed in (table-6). The highest protease activity (835.13±5.04 U/g) was obtained at the temperature of 37^0C. This optimal temperature is similar to those described by Soares V.F.[19] reported optimum protease production by *Bacillus subtilis* under SSF with soy cake at 35°C. However, when the temperature was higher than 50^oC the production was greatly affected due to higher temperature may cause of microorganism respectively.

Table 4
Effect of Inoculum level on protease production from IAS01 under SSF

Inoculum (%)	Total protein (mg/ml)	Protease activity (U/gds)
10	2.47±0.12	652.62±8.96
20	4.11±0.10	750.70±3.08
30	2.79±0.09	713.99±11.90
40	2.80±0.08	722.62±6.79
50	3.29±0.05	639.39±6.91
60	2.82±0.05	603.15±12.65
70	2.45±0.07	572.60±8.75

Values were given as mean ± SD of six experiments in each level of inoculum (ANOVA followed by DMRT) significantly at p≤0.05. Under the culture conditions *viz*. moisture 30%, 72 h incubation at initial pH 7.0, and temperature 30 °C.

Table 5
Effect of pH on protease production from IAS 01 under SSF

pH	Total protein (mg/ml)	Protease activity (U/gds)
4.0	1.35±0.11	492.37±3.26
5.0	2.13±0.14	509.25±2.49
6.0	2.13±0.13	657.92±3.85
7.0	2.84±0.14	687.42±3.85
8.0	4.26±0.13	742.51±1.45
9.0	3.61±0.07	792.21±1.45
10.0	2.41±0.06	722.12±2.07
11.0	2.65±1.08	673.86±7.43
12.0	1.94±1.52	512.76±5.97
13.0	1.84±0.21	439.23±6.54

Values were given as mean ± SD of six experiments in each pH (ANOVA followed by DMRT) significantly at p≤0.05. Under the culture conditions *viz*. moisture 30%, 72 h incubation, 20% inoculum at initial pH 7.0 and temperature 30 °C.

Table 6
Effect of Temperature on protease production from IAS01 under SSF

Temperature (^{O}C)	Total protein (mg/ml)	Protease activity (U/gds)
30	2.47±0.12	760.52±12.96
35	2.79±0.09	795.01±11.35
37	4.11±0.10	835.13±5.04
40	2.80±0.08	815.08±6.66
45	3.29±0.05	738.46±7.55
50	2.82±0.05	784.59±7.83
55	2.45±0.07	660.65±9.40

Values were given as mean ± SD of six experiments in each temperature (ANOVA followed by DMRT) significantly at p≤0.05. Under the culture condition followed with respected moisture 30%, fermentation period 72 h, 20% inoculums and pH 9.0.

Conclusion

Strain "IAS01" could effectively consume the groundnut oil cake and palm oil cake for alkaline protease production under SSF with proper culture condition. According to this way of protease production does not need any extra nutrient for the support of growth. In feature to strongly document for the utilization of agro-industrial by product as raw materials is cheaper cost and more valuable than organic nutrient for alkaline protease production and it is suitable way for large scale protease production at industrial stage.

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