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Optimization of Solid State Fermentation Process Parameters for Improved Production of L-Asparaginase by Endophytic *Fusarium solani*

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ABSTRACT

Aim: To optimize different process parameters for improved production of L-asparaginase by newly isolated endophytic *Fusarium solani* under Solid State Fermentation (SSF) using different agro-industrial wastes. **Place and Duration of Study:** School of Life Sciences, SRTM University, Nanded, Maharashtra, India between April and June -2018. **Methodology:** Production of the L-asparaginase was performed under SSF utilizing different types of agro-industrial wastes as a substrate for the enzyme production. Five different substrates, coconut oil cake, sesame oil cake, corn cob, sugarcane bagasse and wheat bran, were used in this study individually and as a combination (1:1). The substrate that gave the maximum L-asparaginase yield was selected for optimization of the fermentation parameters: particle size, moisture content, pH, inoculum age, inoculum size, temperature, incubation period, and carbon and nitrogen sources. **Results:** Among the different substrates evaluated, coconut oil cake gave maximum L-asparaginase production (8.79 IU/gds) and it was selected for optimization of the fermentation parameters. L-asparaginase maximum yield (13.87 IU/gds) was achieved with the following optimized parameters: coconut oil cake solid substrate with concentration (10 gm), particle size (3 mm), moisture content (70% v/w), acidic pH 6.0, inoculum age (4 days), inoculum size 10^6 spores/ mL, temperature (30 ºC), and incubation period (7 days). 1.9 fold increasing in the L-asparaginase production was achieved by supplementation with 0.1% glucose and 0.1% ammonium chloride. In addition, using mixed substrates of coconut oil cake and wheat bran in 1:1 (w/w) ratio under same optimized conditions showed further enhancement of the L-asparaginase production by 1.2 fold and 19.56 IU/gds enzyme activity was achieved with 2.2 fold final increasing in the production.

1. INTRODUCTION

L-asparaginase (EC 3.5.1.1) is a critical enzyme involved in L-asparagine hydrolysis. It gained well-known significance as a potent chemotherapeutic enzyme used in treating of various forms of lymphomas and lympho-proliferative disorders especially acute lymphoblastic type of leukaemia (ALL) and recently L –asparaginase has been established as an essential constituent in medicine. Cancerous cells discriminate themselves from the normal cells through diminished in L – asparagine expression. Therefore, they are not able to produce L – asparagine and depend exclusively on the plasma circulating L –asparagine [1, 2]. Absolute depletion of plasma circulating L-asparagine affects cancerous cell through leading to protein synthesis rapid inhibition, resulting in apoptosis [3, 4].

Importance of the microorganisms as sources of L-asparaginase was studied thoroughly since first discovery of it from *Escherichia coli* along with its antitumour activity which was revealed in of guinea pigs serum [5]. A lot of researches since that time have comprehensively concerned with searching for alternative sources of L-asparaginase microbial strains producers such as *Erwinia carotovora* [6], *Serratia marcescens* [7]. Eukaryotic sources like *Saccharomyces cerevisiae* yeast [8] and filamentous fungi like *Penicillium*, *Aspergillus,* and *Fusarium* have a potential for the L-asparaginase production [9] with less side effect. L-asparaginase comparative estimation for its potent activity from diverse microbial sources showed that biochemical, therapeutic and enzyme production properties differ with different source of strain.

Massive wastes quantities are produced from agricultural-based industries annually. If these wastes will released directly into the environment without suitable disposal process it may lead to environmental pollution and cause harmful consequence on health of human and animal [10].

Over the past years, using of agro-industrial wastes retained high significance in different bioprocesses because of their high nutrient content accompanied with their low cost. These wastes commonly include oil cakes, molasses, straw, husk, bagasse, fruits and vegetables peels, etc; have been reported for production of different valuable products. Converting these nutritionally rich by-products to useful and valuable bio-products through fermentation processes not just minimizes the production process cost but as well reduce the environmental pollution risk [11].

Submerged Fermentation (SmF) has been traditionally used for the L- asparaginase production though it has many drawbacks. SSF has been developed to overcome the SmF drawbacks and it is now considered as an economical alternative process for the biosynthesis of vast number of bio-products through utilization of the agro-industrial waste [12, 10, 11].

SSF is considered as any biotechnological processes where the organisms are grown on non-soluble materials or the solid substrates with the absence or semi absence of the free water. Though, substrate should have adequate moisture in order to encourage microbial growth and metabolism [10, 13]. SSF is generally using microbes for production of valuable bioactive compounds particularly enzymes, because it uses raw materials with low-cost, generating fewer effluent, and biocatalysts production with novel catalytic characteristics [11].

In the present study, we tried to investigate the possibility of utilizing different agro-industrial waste as substrates and optimizing of various bioprocess parameters for improved production of L-asparaginase by newly isolated endophytic *F. solani*.

2. METHODOLOGY

2.1. Agro-Industrial Waste Substrates

Five different agro-industrial waste substrates; coconut oil cake, sesame oil cake, corn cob, sugarcane bagasse, and wheat bran; were procured from the local markets, Nanded, India. These substrates were dried and ground into smaller particles by blender.

2.1.1 Fungal endophytes strain

In this study, *F. solani* was isolated from *Curcuma longa* rhizome. It was maintained on Potato Dextrose Agar (PDA) slants through regularly sub-culturing on PDA at 28 °C and stored at 4 °C. It was previously screened for L-asparaginase and L-glutaminase production according to the method described by Gulati *et al*. [14] and it showed ability to produce L-asparaginase without glutaminase activity.

2.1.2 Preparation of inoculum

The inoculum was prepared according to the method of Meghavarnam and Janakiraman [15] with some modifications. For preparation of the inoculums, modified Czapek-Dox broth (MCdox) was used [36]. The broth medium was prepared, sterilized and inoculated with *F.*

solani spore suspension (~10^6 spores/ mL). The culture was incubated at 28 °C at static conditions for 72 h. Under sterile conditions, the mycelial mat obtained on the medium was homogenized with water using the mortar and pestle.

2.2 Substrates Preparation and Screening of Production

The selected agro-industrial waste solid substrates, coconut oil cake, sesame oil cake, corn cob, sugarcane bagasse, and wheat bran, were used and prepared for SSF production of L-asparaginase. Ten grams of each one substrate was added separately in a 250 mL Erlenmeyer flask and was moistened using distilled water (60%) containing L-asparagine (0.05% w/w). All flasks were subjected to sterilization by autoclave at 121 °C (15 psi) for 20 min. The flasks were inoculated after completely cooling with 1.0 mL of spore suspension (~10^6 spores/ mL). The flasks contents were mixed thoroughly and incubated at 30 °C for 5 days.

2.2.1 Enzyme extraction

L-asparaginase was extracted from substrates according to the method of Varalakshmi and Raju [16] with some modifications. After 6 days of incubation period, phosphate buffer (0.1 M, pH 8.0) was used for extraction of the crude enzyme from the fermented media. Fermented substrate was mixed gently with 50 ml (1:5 w/v) of the buffer. For optimizing the incubation period parameter; one gram of each substrate containing fungal biomass was taken at regular intervals under aseptic condition into a flask containing 5 mL of the buffer (1:5 w/v). The flasks were kept on a rotary shaker at speed of 150 rpm for 30 min. A muslin cloth was used for separating of the extract by squeezing through it and the filtered by using Whatman No. 1 filter paper. The filtered extract was subjected to centrifugation at 10,000 rpm for10 min at 4 °C in a cooling centrifuge. Supernatant was used for estimation of the enzyme activity.

2.2.2 L-asparaginase activity estimation

Activity of L-asparaginase in the culture filtrates was estimated according to the method of Imada *et al*. [17]. Using Nessler's reagent, the hydrolysis rate of L-asparagine was detected by quantifying the liberating ammonia. A mix of 0.04 M L-asparagine (0.5 mL), enzyme extract (0.5 mL), 0.05 M Tris-HCl buffer of pH 7.2 (0.5 mL) and of distilled water (0.5 mL) was incubated at 37 °C for 30 min. To stop the reaction, 0.5 mL of 1.5 M trichloroacetic acid (TCA) was added to the mixture. The ammonia liberated was determined in the supernatant spectrophotometrically through adding of Nessler's reagent (0.2 mL) into tubes containing 0.1

mL of enzyme and 3.7 mL of distilled water. Blank tubes were prepared by adding the enzyme after the addition of TCA. The tubes were incubated for 20 min at room temperature. The absorbance was measured at 450 nm. For preparation of the standard curve, gradual concentration of ammonium chloride was used. One international unit (IU) of L-asparaginase is the amount of enzyme required to liberate one μmol/min/ml of ammonia at 37 ºC.

2.3 Optimizing of Process Parameters for L-asparaginase Production

For optimization of L-asparaginase production, optimization of different nutritional and physicochemical parameters such as particle size (2 mm, 3 mm and 4 mm), initial moisture content (30%, 40%, 50%, 60%, 70% and 80%), initial pH (4.0 – 10.0), inoculum age (3 days to 7 days), inoculum size (10^5, 10^6, 10^7, 10^8 and 10^9 spores/mL), incubation temperature (28, 30, 35 and 37 °C), incubation period (3 days to 10 days) on L-asparaginase production has been studied. The optimization process was carried out through one factor at a time in a sequential approach for the coconut oil cake substrate which was used as a sole carbon source for L-asparaginase production. In addition, effect of supplementary carbon sources like glucose, lactose, fructose, sucrose, maltose, ribose, cellulose and starch at 0.1% (w/w) and nitrogen sources such as ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate, potassium nitrate, peptone, urea, and yeast extract at 0.1% (w/w) on production of L-asparaginase has been studied. At 24 h intervals, samples were drawn constantly and the activity of the enzyme was detected. All the experiments were carried out in triplicates.

2.4. Mixed Substrates Effect on L-asparaginase Production

Mixed substrate fermentation with various combinations was used for improving of L-asparaginase production using the substrates coconut oil cake, sesame oil cake, corn cob, sugarcane bagasse, and wheat bran. Each one substrate was mixed in ratio 1:1 (w/w) with coconut oil cake substrate which was considered as constant and assessed for production of L-asparaginase. Evaluation of the combination of coconut oil cake and wheat bran was carried out in diverse ratios (1:2, 2:1, 1:1, 4:3 and 3:1) (w/w) for further optimizing of production of L-asparaginase. The fermentation process was performed with the parameters which were optimized during the optimization process. From each combination mixture ten grams was put separately in a 250 mL Erlenmeyer flask and moisture content was set to 70% with distilled water containing L-asparagine (0.05% w/w). Sterilization of the flasks was performed by autoclaving at 121 °C (15 psi) and for 15 min. After completely cooling, each one of the flasks

was inoculated with 1.0 mL of spore suspension (10^6 spores/ mL). The mixture in the flasks were mixed properly and incubated at 30 °C for 7 days.

2.5 Statistical Analysis

All the experiments were carried out in triplicates and the results were expressed as mean ±SD using Minitab statistical software (version 18.0). The statistical significance between mean values was accessed by ANOVA through Tukey at significance level of ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1 Various Agro-Industrial Wastes Screening for L-asparaginase Production by *F. Solani*

Recently, agro-industrial waste products utilization has attained significance in bioprocess industries since they are rich in high nutrient content and little cost [18]. Using of agro-industrial waste in fermentation reduces the cost of fermentation process and significantly reduces the environmental pollution risk [19]. Solid substrate fermentation considered as a low cost process for the enzymes production since it gives enhanced process control, high yields and several other favourable aspects [15]. In this study, different agro-industrial waste substrate; coconut oil cake, sesame oil cake, corn cob, sugarcane bagasse , and wheat bran; were screened for the production of L-asparaginase by *F. solani*. All the substrates were positive for the L-asparaginase production with yields ranging from 1.60 to 8.79 (IU/gds) (Table.1).

Table.1 L-asparaginase activity in *F. solani* using different agro-industrial wastes solid substrates

Substrate	Enzyme Activity IU/gds
coconut oil cake	8.79±0.16
sesame oil cake	4.17±0.149
wheat bran	4.53±0.23
sugarcane bagasse	1.76±0.14
corn cob	1.60±0.19

IU/gds= International Units/gram dry substrate. Given data are the Mean± S.D (n=3)

Among all the substrate, coconut oil cake showed better growth support and also gave maximum enzyme activity of 8.79 (IU/gds) therefore, coconut oil cake might be considered as potential substrate for the L-asparaginase production under SSF process and that results agree with the previous work with *Serratia marcescens* (NCIM 2919) [19], *Aspergillus melleus* AVNR-1 [18] which reported that the use of coconut oil cake is considered better for the L-asparaginase production. Some reports also showed that coconut oil cake could be used as a potential substrate for the production of different enzymes like α-amylase [20, 21], lipase [12] and L-glutaminase [22]. Coconut oil cake is high rich in protein and is used in for farm animals feeding ingredients [21, 23]. From our results, it is clear that coconut oil cake might be a potential substrate used in SSF for L-asparaginase production.

3.2. Effect of Substrate Particle Size

Present study showed that the L-asparaginase maximum production of (8.89 IU/gds) was found when particle size was (3 mm) of coconut oil cake [Fig.1]. That finding was in accordance with the result reported by Meghavarnam and Janakiraman using soybean meal substrate [15]. Particles sizes (2 mm) and (4 mm) caused reduction in production of L-asparaginase which might be related to the influences on the surface area and diffusion of oxygen [24, 25].

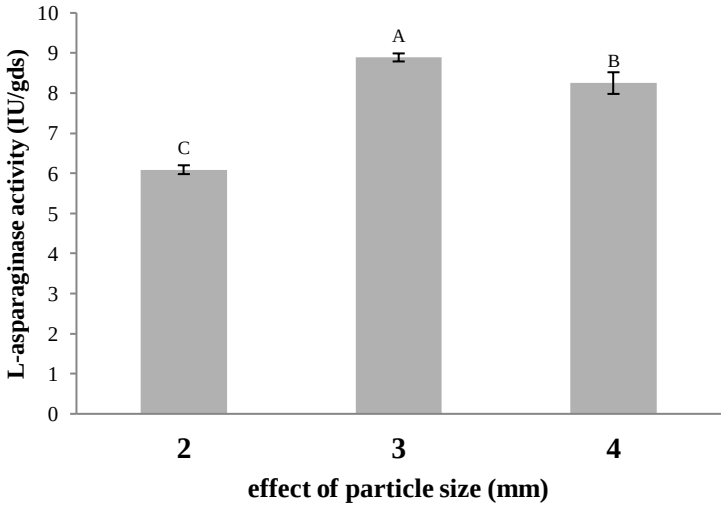

Fig. 1. Effect of substrate particle size on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

3.3 Effect of the Initial Moisture Content

Initial moisture content considered one of the most crucial parameters in the SSF. In the present study, the maximum enzyme production (9.18 IU/gds) was revealed in fermentation condition with 70% moisture content [Fig. 2]. Same finding was reported in *Fusarium culmorum* (ASP-87) by Meghavarnam and Janakiraman [15], *Aspergillus niger* by Mishra [26], *Fusarium equiseti* by Hosamani and Kaliwal [27] and *Aspergillus terreus* MTCC 1782 by Varalakshmi and Raju [16]. Moisture content beyond and less than that level found to cause reduction in the enzyme activity. The reasons behind that might be due to the reduction in substrate poreicity with increasing in anaerobic conditions which could lead to contamination with bacteria in case of higher moisture content and decreasing in solubility of substrate in case of less moisture cintent [28, 15, 11].

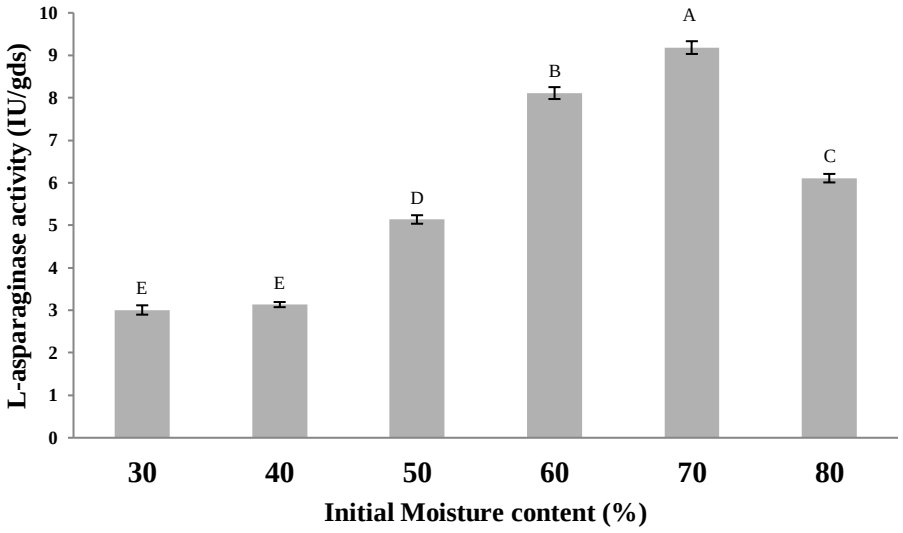

Fig. 2. Effect of initial moisture content on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

3.4 Effect of Initial pH

In the fermentation medium, the pH and temperature are the most important physical parameters that support the growth and the formation of the product by controlling the components transport through the cell membrane [29, 15]. However, in SSF only the initial pH of the medium could be adjusted prior to the inoculation [30]. In the present study, maximum L-asparaginase production (9.48 IU/gds) was revealed when initial pH 6.0 was employed [Fig.

3]. That is going with the fact that fungi prefer acidic pH [30]. Same finding was reported by and Pallem in *Fusarium oxysporum* (NCIM 1008) using corn husk as a substrate [11]. Increasing or decreasing initial pH away from the optimum value gives less enzyme activity which could be related to enzyme denaturation [15]. On the contrary, Sameera and Raju [22] reported initial pH of 7.0 as optimum pH for L-asparaginase production in *Aspergillus wentii* MTCC 1901 using mixed substrate of coconut oil cake and sesame oil cake.

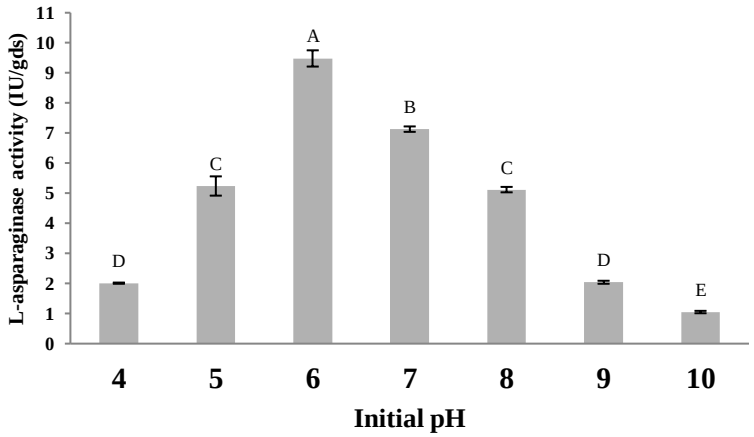

Fig. 3. Effect of initial pH on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

3.5 Effect of Inoculum Age and Inoculum Size

SSF was conducted using spore inoculum of different ages (3-7 day old culture) to study the inoculum age effect on the production of L-asparaginase. Maximum production (9.66 IU/gds) was revealed with inoculum from the day 4 old culture [Fig. 4]. It was observed that the production activity was decreased with increasing the inoculum age which might be related to the viable cells number [31]. Varalakshmi and Raju [16] reported maximum L-asparaginase yield by the inoculum age of five days by *Aspergillus terreus* MTCC 1782 when Bajra seed flour substrate was used. Pallem *et al*. [32] reported 7 day old culture for maximum production of L-asparaginase by *Fusarium oxysporum* using wheat bran as a SSF substrate.

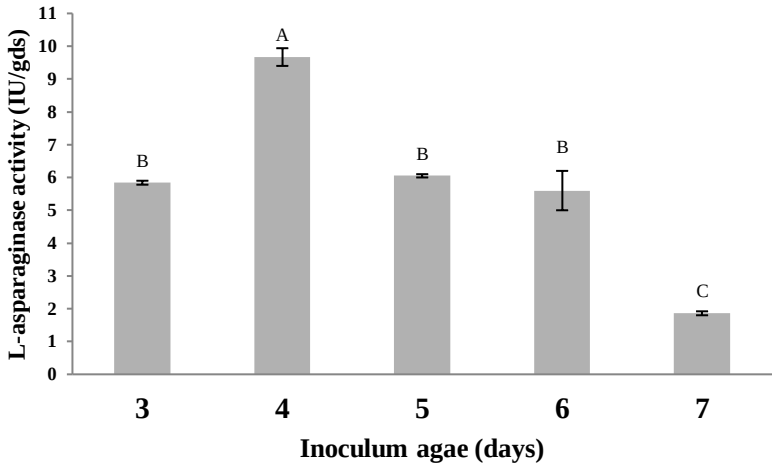

Fig. 4. Effect of inoculum age on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

In SSF, inoculum size has a major role in the enzymes production. In the present study, L-asparaginase production was found with the inoculum size 10^6 spores/mL (10.07 IU/gds) [Fig. 5]. Inoculum size beyond or lower than level significantly lower the enzyme production. This might be because of fast nutrients depletion in higher inoculum size causing reduction in metabolic activity or may be related to the phenomenon in mycology: "the spore germination self-inhibition" in which the spores contain a pre-packaged self-inhibitor which prevents germination in crowded conditions. Whereas in case of less inoculum size, more time is required in order to initiate a microbial growth and subsequently utilizing the substrate to produce the enzyme. Another possible interpretation is that fungi under small inoculum sizes noticed to produce a stage of transient mycelia in which the length of mycelium is inversely proportional to the size of inoculum [33]. Meghavarnam and Janakiraman reported inoculum size of 10^8 spores/mL for maximum L-asparaginase production by *Fusarium culmorum* (ASP-87) using soybean meal as a supporting substrate [15].

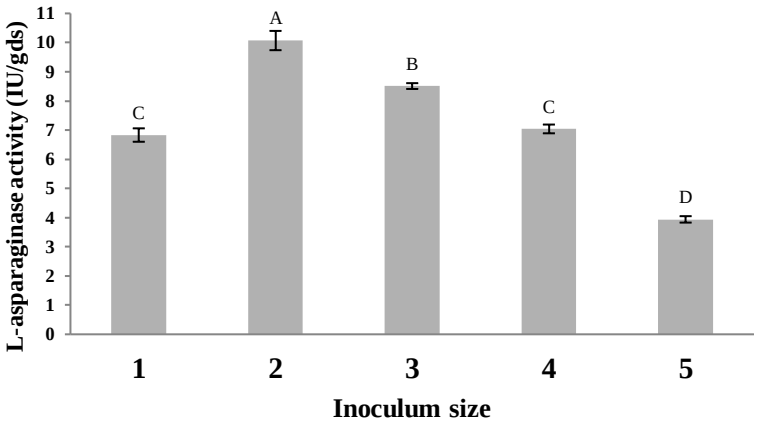

Fig. 5. Effect of inoculum size on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

3.6 Effect of Incubation Temperature

In the present study, L-asparaginase production was found to increase gradually from 28 to 30 °C with maximum (10.98 IU/gds) at 30 °C [Fig. 6]. Enzyme production activity was shown to be less with higher incubation temperatures. Temperature affects the chemical reaction rate and integrity of protein structure consequently affects the enzymatic activity. However, each enzyme have a different range of the thermal stability and further than it, their denaturation occurs [34]. Accordingly, same finding was reported by Pallem *et al*. [32] and Meghavarnam and Janakiraman [15] in *Fusarium oxysporum* and *Fusarium culmorum* (ASP-87) respectively.

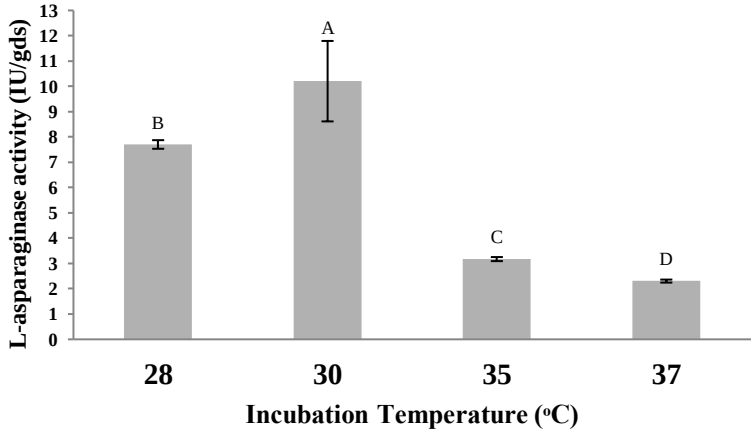

Fig.6. Effect of incubation temperature on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

3.7 Effect of Incubation Period

In the present study, enzyme activity of *F. solani* was tested periodically from 3 to 10 days of incubation. The maximum production of L-asparaginase (11.74 IU/gds) was found on the day 7 of the incubation after which the production was markedly declined [Fig. 7]. The decline in the production upon increasing the incubation period might be due to either enzyme denaturation [35] or due to nutrients depletion which required for the enzyme production [20]. Supriya *et al*. has reported maximum production of the enzyme by *Aspergillus melleus* AVNR-1 on the day 7 of incubation which is in accordance with our findings [18]. In contrary, Meghavarnam and Janakiraman has reported highest production of enzyme from *F. culmorum* (ASP-87) on the day 6 of incubation [15].

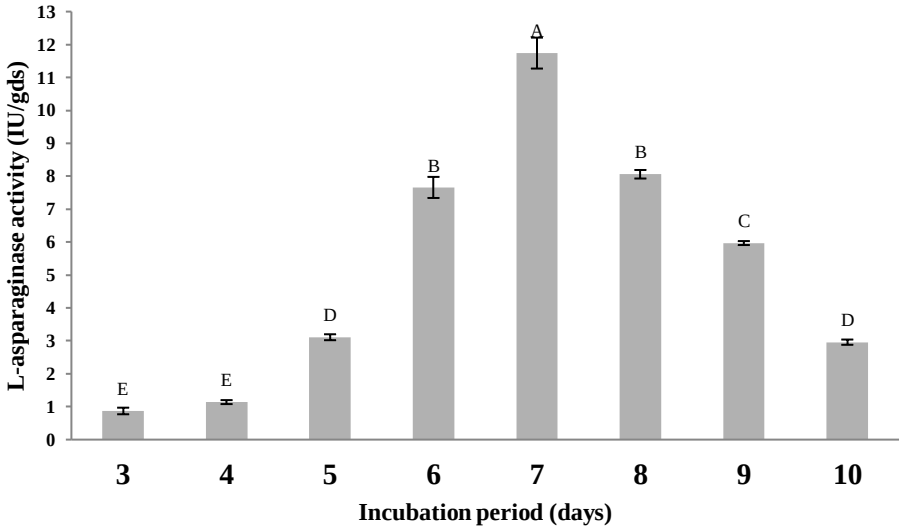

Fig.7. Effect of incubation period on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

3.8 Effect of Supplementation with Carbon and Nitrogen Sources

Among the different carbon and nitrogen sources supplemented to coconut oil cake substrate at 0.1% (w/w), the glucose as a supplemented carbon source and ammonium chloride as a supplemented nitrogen source has observed to show noticeable impact on the L-asparaginase production (12.94 IU/gds and 16.77 IU/gds respectively) [Fig. 8 & 9]. Many reports has shown that supplementation with glucose optimize L-asparaginase production [15, 32, 16]. Regarding nitrogen sources supplementation, Meghavarnam and Janakiraman reported that ammonium

chloride has a positive effect on optimization of L-asparaginase production and this finding is in accordance with our findings [15]. In contrary, ammonium sulphate and urea were reported by Varalakshmi and Raju [16] and Pallem [11] to be the best nitrogen source supplemented in SSF to maximize L-asparaginase production.

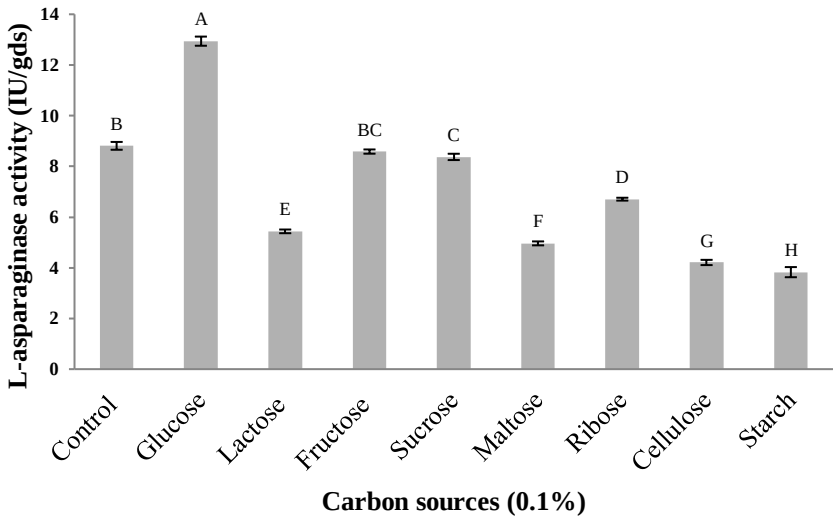

Fig.8 Effect of carbon source supplementation on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

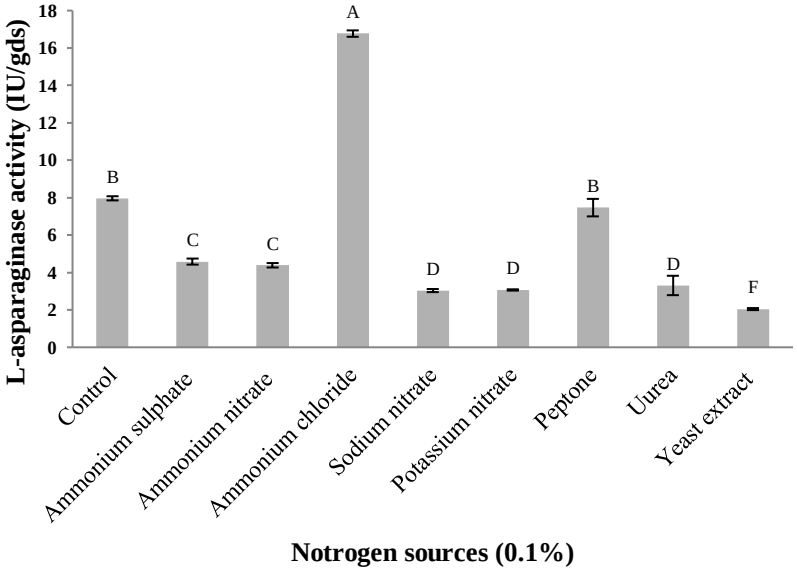

Fig.9 Effect of nitrogen source supplementation on L-asparaginase production by *F. solani* under SSF. In each series, Means that do not share a letter are significantly different (at *P<0.05*) according to Tukey.

3.10 Mixed Substrates Effect on L-asparaginase Production

Maximum L-asparaginase activity was revealed when coconut oil cake was mixed with wheat bran (19.56 IU/gds) comparing with individual substrate [Table. 2]. Meghavarnam and Janakiraman [15] reported maximum production of L-asparaginase by *F. culmorum* (ASP-87) using soybean meal mixed with wheat bran in ratio 1:1. Sameera and Raju reported L-asparaginase maximum yield from *Aspergillus wentii* MTCC 1901 by using mixed substrate composition 1.25:3.75 of coconut oil cake and sesame oil cake as a solid substrate [22].

Table.2 Grouping Information off mixed substrates affecting L-asparaginase production using the Tukey Method and 95% Confidence

Factor	n	Mean of Enzyme Activity IU/gds	Grouping		
Coconut oil cake+ Wheat bran	3	19.56±0.50	A		
Coconut oil cake + Sesame oil cake	3	11.17±0.72		B	
Coconut oil cake + Sugarcane bagasse	3	10.37±0.63		B	C
Coconut oil cake + Corn cob	3	9.99±0.18			C

IU/gds= International Units/gram dry substrate. Given data are the Mean± S.D (n=3), *Means that do not share a letter are significantly different.*

4. CONCLUSION

Agro-industrial wastes composed of high level of nutrient and are rich in bioactive compounds which could be considered as a low cost raw materials used for production of different valuable products through fermentation processes by different microorganisms. In the other hand, utilization of these wastes reduces the environmental pollution risk. L-asparaginase is considered one of the most important enzymes used in the treatment protocols of human cancers and principally produced commercially using bacteria through SmF which has many drawbacks such as high production cost and many side effects associated with using of L-asparaginase of prokaryotic sources. Production of L-asparaginase through SSF especially by fungi, i.e. eukaryotic source, could lead to overcome these drawbacks. In the present study, we tried to design a process of SSF utilizing different agro-industrial wastes as low cost raw materials for production of L-asparaginase using *F. solani*. Findings in the present study are promising and must be considered in any future research on production of L-asparaginase and may be exploited in the large scale production.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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