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Solid state fermentation: An effective fermentation strategy for the production of L-asparaginase by *Fusarium culmorum* (ASP-87)

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ABSTRACT

Production of L-asparaginase by *Fusarium culmorum* (ASP-87) isolated from tropical soil was investigated under solid state fermentation on a laboratory scale using sixty five (65) agro based materials. Among the different agro based materials evaluated, soybean meal supported maximum L-asparaginase production (7.21 U/gds). Various optimization strategies for the production of L-asparaginase were also carried out with soybean meal and it was observed that inoculum size of 1×10^8 spores/mL, day 6 of incubation period, 3 mm of particle size of the substrate, moisture content of 70%, initial pH of 7.0 and temperature at 30 °C were found to be optimal for L-asparaginase production. Supplementation of glucose (0.1%) and ammonium chloride (0.1%) enhanced L-asparaginase production to 1.7 fold. Mixed substrate fermentation using soybean meal and wheat bran in the ratio (1:1 w/w) further enhanced production of L-asparaginase to 0.5 fold with a final yield of 18.91 U/gds.

1. Introduction

Enzymes are proteins with biocatalytic activity that catalyze and speed up rate of various chemical reactions with substrate specificity by lowering the activation energy. Microbes are widely exploited and are the potential source of industrially important enzymes as they synthesize and secrete large amounts of extracellular enzymes. Fungal enzymes account for approximately 60% of commercially available enzymes. Enzymes produced by fungi are widely used in paper and pulp industries, leather industries, textiles, detergents, food and beverages and therapeutics. The therapeutic enzymes produced by fungi have been commonly used as anti-inflammatory, thrombolytics or anticoagulants and oncolytics as replacement for metabolic deficiency. A major application of therapeutic enzymes is in the chemotherapy of cancer.

L-asparaginase (L-asparagine amido hydrolase E.C.3.5.1.1) is an enzyme belonging to an amidase group which catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. It is widely used as a therapeutic agent for the treatment of acute lymphocytic leukemia (ALL) (mainly in children), Hodgkin's disease, acute myelocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment and melano sarcoma (Stecher et al., 1999; Verma et al., 2007). L-asparaginase also has significant applications in food industry to reduce the formation of carcinogenic acryl amides in deep fried potato (Pedreschi et al., 2008).

L-asparaginase is widely distributed among plants, animals and microbes. However, L-asparaginase from fungal source has gained much attention because of its stability, high productivity and easy cultural conditions.

Industrial production of L-asparaginase by bacteria and fungi has been previously achieved by submerged fermentation (SmF). Commercial production of L-asparaginase is carried out by using *Escherichia coli* and *Erwinia chrysanthemi*. Submerged fermentation has several disadvantages like huge volume of waste water generation and difficulties in effluent treatment process. (Datar, 1986). Solid state fermentation (SSF) has gained much interest during recent years and has emerged as an economical, alternative cost effective process for enzyme production by utilizing agricultural and agro industrial residues as substrates which are converted into products with high commercial value (Pandey, 1992). The use of SSF for production of enzymes has many advantages over submerged fermentation such as less production cost with better physiological properties, smaller reactor volume and cheaper fermentation media, higher production rate and easier downstream processing (Pandey et al., 1999; Bhargav, 2008). Solid substrates, mainly agro based materials are reportedly being utilized as substrates for the production of L-asparaginase (Swathi et al., 2014; Kumar et al., 2013; Hymavathi et al., 2009; Mishra, 2006; El-Bessoumy et al., 2004). These substrates act both as physical support and source of nutrients which influences enzyme production. Therefore, screening and selection of an appropriate substrate is an important step for commercial production of an enzyme.

We have previously reported the production strategy of L-

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asparaginase in *F. culmorum* (ASP-87) by submerged fermentation (Meghavarnam and Janakiraman, 2015). In the present study, we explore the possibility of using agro based materials as sole substrates for the better production of L-asparaginase by *F. culmorum* (ASP-87).

2. Materials and methods

2.1. Substrates and chemicals

Different agro based substrates were procured from mills and few substrates were purchased from the local market in Bengaluru, Karnataka, India. These substrates were dried and ground into smaller particles in a mill and the particle size of 1 mm was used for the experiment. Other media components used in the experiment were obtained from Hi-media (Mumbai, India). All the chemicals were of analytical reagent grade.

2.1.1. Microorganism and culture conditions

The fungus *F. culmorum* strain (ASP-87) used in the study was isolated from tropical soil. This fungus was tested for mycotoxin production and found to be negative for fumonisins and trichothecenes. The fungal culture was maintained on Potato Dextrose Agar (PDA) slants by periodically sub-culturing on PDA at 30 °C and stored at 4 °C.

2.1.2. Inoculum preparation

The culture medium used for inoculum preparation was modified Czapek-Dox medium containing g/L of, Glucose,2.0; L-asparagine,10.0; KH_2PO_4,1.52; KCL,0.52; $MgSO_4{\cdot}7H_2O$,0.52; $CuNO_3{\cdot}3H_2O$,trace; $ZnSO_4.7H_20$,trace; $FeSO_4.7H_20$,trace; pH 7.5 (Saxena and Sinha, 1981). Modified Czapek-Dox broth was prepared, sterilized and inoculated with *F. culmorum* (ASP-87) spore suspension (10^7 spores/mL). The culture was incubated at 30 °C under static cultivation conditions for 96 h. The mycelial mat obtained was homogenized with water using mortar and pestle under sterile conditions and used as inoculum for further experiments.

2.2. Screening and preparation of solid substrates

In total, sixty five (65) types of agro based materials were used. They are arecanut peel, banana peel, banana stem, barley, black gram husk, bengal gram husk, broken chana, broken red rice, broken wheat, coconut fiber, coconut oil cake, carob pod, coffee husk, colocasia, corn cob, corn flakes, corn flour, corn kernel, corn peel, cotton seed oil cake, dry copra waste, flat bean seed, flat bean peel, gram flour waste, green gram husk, green peas waste, groundnut oil cake, groundnut waste, groundnut shell, jack fruit seeds, jowar husk, lemon peel, linseed powder, musambi peel, mushroom, orange peel, paddy husk, paddy straw, pea husk, pea peel, pomegranate peel, potato peel, ragi bran, ragi flour, ragi straw, rajma seed, red gram husk, rice flour, rice husk, saw dust, seri waste, sesame oil cake, sooji, sorghum milling waste, soybean broken, soybean meal, sugarcane bagassae, sunflower oil cake, sweet potato, tamarind peel, tapioca, tea waste, urad dhal waste, wheat bran and wheat flour were screened for L-asparaginase production.

Five grams of each substrate was weighed separately in a 100 mL Erlenmeyer flask and moistened with distilled water (70%) containing L-asparagine (0.05% w/w). The flasks were sterilized by autoclaving at 121 °C (15 psi) for 15 min. After cooling, the flasks were inoculated with 1.0 mL of spore suspension (10^7 spores/mL). The contents of the flasks were mixed thoroughly and incubated at 30 °C for 6 days (Kumar et al., 2013).

2.2.1. Extraction of the enzyme

After 6 days of incubation, L-asparaginase was extracted from substrates according to the method of Kumar et al. (2013). The substrate with fungal biomass was air dried under shade and fifty milliliter of water was added and kept on orbital shaker at (150 rpm) for 30 min. To optimize incubation period 1g of substrate with fungal biomass was withdrawn periodically at regular intervals in aseptic condition and air dried under shade and taken in a beaker containing 10 mL of distilled water (1:10). The contents of flasks were kept on orbital shaker (150 rpm) for 30 min. The extract was separated by squeezing through a muslin cloth and filtered through Whatman No. 1 filter paper. The filtered extract was centrifuged at 10,000 rpm for 10 min. The resultant supernatant was used as enzyme source for L-asparaginase assay.

2.2.2. Determination of L-asparaginase activity

L-asparaginase activity in the culture filtrates was determined by the method of Imada et al. (1973). The L-asparagine hydrolysis rate was determined by quantifying the ammonia liberated using Nessler's reagent. A blend of 0.5 mL of 0.04 M L-asparagine, 0.5 mL of enzyme extract, 0.5 mL of 0.05 M Tris-HCl buffer (pH 7.2) and 0.5 mL of distilled water was incubated at 37 °C for 30 min and 0.5 mL of 1.5 M trichloroacetic acid (TCA) was added to stop the reaction. The ammonia liberated in the supernatant was determined spectrophotometrically by adding 0.2 mL of Nessler's reagent into tubes containing 0.1 mL of enzyme and 3.7 mL of distilled water and incubated at room temperature for 20 min. The absorbance was measured at 450 nm and L-asparaginase activity was expressed as the amount of ammonia liberated per mL per minute under the standard assay conditions.

2.3. Optimization studies for L-asparaginase production

Soybean meal was used as the sole source of carbon for L-asparaginase production. The optimization studies were carried out by one factor at a time (OFAT) approach by varying only a single factor at a time and keeping the remaining factors constant. The effect of various physiological and nutritional parameters such as incubation period (4 days to 9 days), initial moisture content of the substrate (40%, 50%, 60%, 70%, 80% and 90%), particle size fine (2 mm), intermediate (3 mm) and coarse (4 mm), inoculum size (10^6, 10^7, 10^8 and 10^9 spores/mL), initial pH 4.0–9.0), incubation temperature (25, 28, 30, 33, 35 and 37 °C) on L-asparaginase production has been studied. The influence of supplementation of additional carbon sources such as glucose, maltose, lactose, sucrose, fructose, starch, cellulose, xylose and ribose at 0.1% (w/w) and nitrogen sources such as ammonium chloride, ammonium nitrate, ammonium sulfate, potassium nitrate, sodium nitrate, urea, peptone, yeast extract, beef extract and tryptone at 0.1% (w/w) on L-asparaginase production has been studied. Samples were drawn continuously at 24 h intervals and the enzyme activity was determined. All the experiments were carried out in triplicates.

2.4. Effect of combination of mixed substrates on L-asparaginase production

The substrates soybean meal, carob pod, corn cob, corn kernel, gram husk, green peas, groundnut oil cake, jowar husk, mushroom powder, soybean flour, sugarcane bagassae, sunflower oil cake, urad dhal waste and wheat bran were used for mixed substrate fermentation in various combinations for enhanced production of L-asparaginase. Soybean meal was kept as constant and each substrate was mixed with soybean meal in 1:1 (w/w) ratio and assessed for L-asparaginase production. The substrate combination of soybean mean and wheat bran was evaluated in different ratios (1:4, 2:3, 1:1, 3:2 and 4:1) (w/w) for L-asparaginase production. The fermentation was carried out with the parameters that were optimized by OFTA approach. Five grams of each combination of substrates was weighed separately in a 100 mL Erlenmeyer flask and moistened with distilled water (70%) containing L-asparagine (0.05% w/w). The flasks were sterilized by autoclaving at 121 °C (15 psi) for 15 min. After cooling, the flasks were inoculated with 1.0 mL of spore suspension (10^8 spores/mL). The contents of the flasks were mixed thoroughly and incubated at 30 °C for 6 days.

2.5. Statistical analysis

All the experiments were carried out in triplicates and the results were expressed as mean ± SD using SPSS software (version 20.0). The statistical significance between mean values was accessed by ANOVA through Duncan's Multiple Range Test (DMRT) at significance level of ($p < 0.05$).

3. Results and discussion

3.1. Screening of various agro based materials for L-asparaginase production in F. culmorum (ASP-87)

Solid substrate fermentation is a low cost method for the production of enzymes as it provides better process control, higher yields besides several other beneficial aspects (Prabhakar, 2005). Selection of appropriate substrates for the production of enzymes is crucial in solid substrate fermentation as it depends on several factors mainly related with the cost, nutritive value and availability of the substrate (Pandey et al., 2000). Owing to its importance in pharmaceutical as well as in food industries, there has been a paramount interest in developing methods for cheaper production of this enzyme. One alternative low cost method for the production of any enzyme is the use of SSF mainly using agricultural based materials as substrates. In our present study, a total of sixty five (65) agro based materials were used as substrates or as carbon source for L-asparaginase production by *Fusarium culmorum* (ASP-87) under SSF. Of the sixty five (65) various substrates used, soybean meal proved to be the most suitable substrate for growth and maximum production of L-asparaginase (7.21 U/gds) followed by sugarcane bagassae (6.95 U/gds), corn kernel (6.60 U/gds), wheat bran (6.28 U/gds), corn cob and carob pod (6.16 U/gds) (Table 1). Our results were in accordance with the observations made with *Aspergillus* sp. ALAA2000 (Abbas Ahmed et al., 2015), *Fusarium equiseti* (Hosamani and Kaliwal, 2011), *Streptomyces* sp. (Basha et al., 2009) and *Pseudomonas aeruginosa* 50071 (El-Bessoumy et al., 2004) which preferred soybean meal for the better production of L-asparaginase. Minimum production of L-asparaginase (0.66–0.41 U/gds) was assessed when arecanut peel, coconut fiber, gram flour waste, paddy husk, sorghum milling waste and wheat flour waste were used as substrates (Table 1). Overall, we observed that most of the substrates supported the growth and enzyme production under SSF but, however, with exception of few substrates such as banana peel, banana stem, saw dust and seri waste. This might be due to inappropriate physical properties such as particle size, geometry and compactness of the substrate (Krishna, 2005). Other substrates such as wheat bran, rice bran, sugarcane bagassae, rice husk, red gram husk, green gram husk, bengal gram husk, coconut oil cake, groundnut oil cake, cotton seed oil cake, corn cob, carob pod, paddy straw, corn pith, and tea waste have also been reported by different workers as suitable substrates for the production L-asparaginase (Elshafei and El-Ghonemy, 2015; Swathi et al., 2014; Ghosh et al., 2013; Makky, 2013; Kumar et al., 2013; Pallem et al., 2011; Hymavathi et al., 2009; Mishra, 2006). Swathi et al. (2014), Kumar et al. (2013) and Pallem et al. (2011) reported wheat bran as the most suitable substrate for increased yield of L-asparaginase in *Beauveria bassiana* (MSS 18/41), *Cladosporium* sp. and *Fusarium oxysporum* respectively. However, our study categorically revealed that soybean meal as the most suitable substrate for the enhanced production of L-asparaginase. Soybean meal is nothing but a byproduct in soybean processing industries, therefore as a carbon source may reduce the cost of the enzyme production. It is also used as a medium for the production of enzymes such as lipases (Vargas et al., 2008), xlyanases (Maciel et al., 2008), cellulases (Vitcosque et al., 2012) and polygalacturonase (Coffman et al., 2014). Moreover, the biochemical composition of soybean meal i.e. high protein (48–60%) and low lignin content makes

Table 1
List of solid substrates (agro based materials) used for L-asparaginase production in *Fusarium culmorum* (ASP-87).

Sl. no.	Substrate	Enzyme activity (U/gds)	Sl. no.	Substrate	Enzyme activity (U/gds)
1	Arecanut peel	0.66 ± 0.28	34	Musambi peel	1.91 ± 0.14
2	Banana peel	NG	35	Mushroom powder	4.16 ± 0.19
3	Banana stem	NG	36	Orange peel	2.5 ± 0.25
4	Barley	1.48 ± 0.27	37	Paddy husk	0.54 ± 0.07
5	Black gram husk	4.25 ± 0.25	38	Paddy straw	1.33 ± 0.38
6	Bengal gram husk	3.33 ± 0.28	39	Pea husk	2.08 ± 0.14
7	Broken chana	1.91 ± 0.14	40	Pea peel	3.25 ± 0.25
8	Broken red rice	2.62 ± 0.12	41	Pomegranate peel	3.58 ± 0.38
9	Broken wheat	3.16 ± 0.14	42	Potato peel	3.68 ± 0.16
10	Coconut fiber	0.45 ± 0.19	43	Ragi bran	1.33 ± 0.38
11	Coconut oil cake	1.91 ± 0.14	44	Ragi flour	0.58 ± 0.14
12	Carob pod	6.16 ± 0.28	45	Ragi straw	1.16 ± 0.28
13	Coffee husk	1.08 ± 0.38	46	Rajma seed	1.33 ± 0.14
14	Colocasia	1.59 ± 0.37	47	Red gram husk	3.0 ± 0.25
15	Corn cob	6.16 ± 0.14	48	Rice flour	1.25 ± 0.25
16	Corn flakes	3.08 ± 0.14	49	Rice husk	1.83 ± 0.38
17	Corn flour	2.45 ± 0.19	50	Saw dust	NG
18	Corn kernel	6.60 ± 0.12	51	Seri waste	NG
19	Corn peel	2.41 ± 0.38	52	Sesame oil cake	2.66 ± 0.28
20	Cotton seed oil cake	2.91 ± 0.14	53	Sooji	2.12 ± 0.45
21	Dry copra waste	2.36 ± 0.37	54	Sorghum milling waste	0.41 ± 0.14
22	Flat bean seed	4.25 ± 0.25	55	Soybean flour	5.16 ± 0.14
23	Flat bean peel	3.08 ± 0.38	56	Soybean meal	7.21 ± 0.10
24	Gram flour waste	0.41 ± 0.14	57	Sugarcane bagassae	6.95 ± 0.19
25	Green gram husk	3.37 ± 0.12	58	Sunflower oil cake	5.60 ± 0.20
26	Green peas waste	5.58 ± 0.14	59	Sweet potato	3.33 ± 0.38
27	Groundnut oil cake	4.33 ± 0.28	60	Tamarind peel	2.66 ± 0.28
28	Groundnut waste	3.16 ± 0.52	61	Tapioca	3.11 ± 0.12
29	Groundnut shell	2.58 ± 0.14	62	Tea waste	1.08 ± 0.38
30	Jack fruit seeds	3.25 ± 0.25	63	Urad dhal waste	5.54 ± 0.07
31	Jowar husk	5.08 ± 0.14	64	Wheat bran	6.28 ± 0.30
32	Lemon peel	2.16 ± 0.14	65	Wheat flour	0.58 ± 0.08
33	Linseed oil cake	1.58 ± 0.14			

NG- No growth, U/gds = Units/gram dry substrate. Data given are the mean of three replicates ± standard error ($P \leq 0.05$).

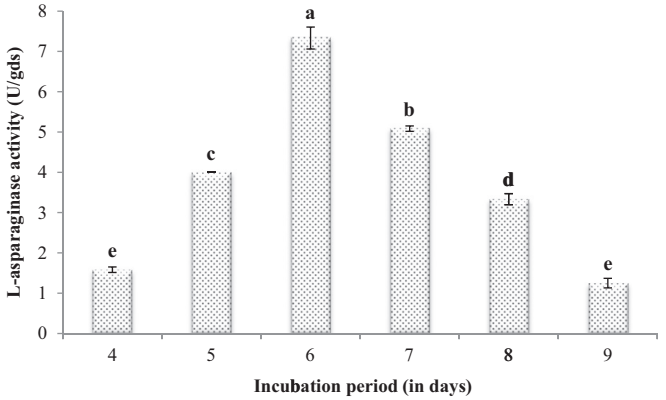

Fig. 1. Effect of incubation period on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. In each series, mean values represented with the same lower case alphabets (a–e) are not significantly different (at $P < 0.05$) according to DMRT.

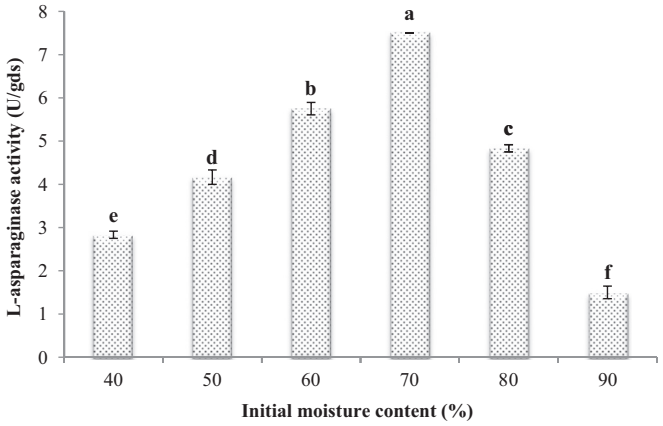

Fig. 2. Effect of initial moisture content on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. In each series, mean values represented with the same lower case alphabets (a–f) are not significantly different (at $P < 0.05$) according to DMRT.

it as an ideal substrate for the growth and production of L-asparaginase. Further studies were carried out with soybean meal as a substrate.

3.2. Effect of incubation period

Effect of fermentation period on L-asparaginase production by *F. culmorum* (ASP-87) in solid state fermentation was tested from 4 to 9 days of incubation with periodic testing of enzyme activity. Results of the present study revealed L-asparaginase production increased exponentially with increase in incubation period and attained maximum (7.33 U/gds) on the 6th day. However, further increase in incubation time gradually declined L-asparaginase production (Fig. 1). This might be because of the denaturation of the enzyme brought by the interaction with other constituents in the medium (Ramesh and Lonsane, 1987) or due to the depletion of nutrients that stimulates the production of secondary metabolites, resulting in lower yield of enzyme (Francis et al., 2002; Ramachandran et al., 2004). In our study, maximum production of L-asparaginase was obtained on day 6, whereas, Mishra (2006) and Kumar et al. (2013) reported maximum L-asparaginase production in *Aspergillus niger* and *Cladosporium* sp. on day 4 and 5 respectively.

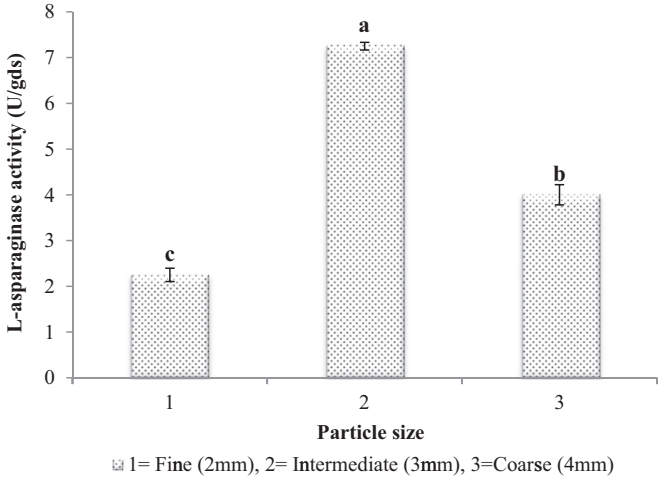

Fig. 3. Effect of particle size of substrate on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. In each series, mean values represented with the same lower case alphabets (a–c) are not significantly different (at $P < 0.05$) according to DMRT.

3.2.1. Effect of initial moisture content, particle size of substrate and inoculum size

Among the several factors that are important for microbial growth and enzyme production under SSF, initial moisture content of the substrate, particle size of the substrate and size of the inoculum are the most critical factors that influence the growth, biosynthesis and secretion of different metabolites (Pandey et al., 2000; Ellaiah, 2002). The maximum L-asparaginase production (7.33 U/gds) was observed at 70% moisture content as reported in *Aspergillus niger* and *Fusarium equiseti* on different solid substrates (Mishra, 2006; Hosamani and Kaliwal, 2011). Further increase in moisture content of the fermentation medium however, led to the reduction of L-asparaginase production. This could be due to decrease in the porosity and agglomeration of particles, thereby limiting the oxygen transfer and therefore high vulnerability to bacterial contamination (Pandey, 2003; Hamidi-Esfahani, 2004; Sandhya, 2005). The activity of L-asparaginase decreased at lower (40%) moisture content which may be due to reduced solubility of nutrients present in the solid substrate, lower degree of swelling and restriction of fungal growth thereby decreasing the enzyme yield (Fig. 2).

In SSF process, the availability of surface area of the substrate particle plays a vital role for microbial attachment, mass transfer of nutrients and enzyme production. L-asparaginase production was maximum (7.16 U/gds) with intermediate particle size (3 mm) of soybean meal (Fig. 3). Whereas, fine (2 mm) and coarse (4 mm) particle size resulted in decreased L-asparaginase production. This may be due to intra particulate associated aeration and limited surface area available for microbial attachment. Similar such observations were also made by Hosamani and Kaliwal (2011) in *Fusarium equiseti* when soybean meal was used as substrate.

In SSF process, the size of inoculum plays a significant role in the production of enzymes. We observed that L-asparaginase production increased with increase in inoculum size upto 1×10^8 spores/mL (7.25 U/gds) and further increase resulted in decreased enzyme production, although the growth was at maximum (Fig. 4). This could be due to depletion of nutrients within a shorter period, which would result in decreased metabolic activity (Kashyap et al., 2002). In general, lower inoculum size requires longer time for the cells to multiply and may not be sufficient for initiating microbial growth and enzyme production, whereas an increase in inoculum size would ensure rapid proliferation of biomass and subsequent enzyme synthesis (Sabu et al., 2005).

3.2.2. Effect of initial pH and temperature

Among the physical parameters, pH and temperature of the fermentation medium are the most critical parameters affecting the transport of various components across the cell membrane which in turn support the cell growth and product formation (Pandey et al., 2000; Krishna, 2005; Kapoor et al., 2008). However, in solid-state fermentations the pH is never controlled during fermentation, only the initial pH of the substrate is adjusted before inoculation. The optimum

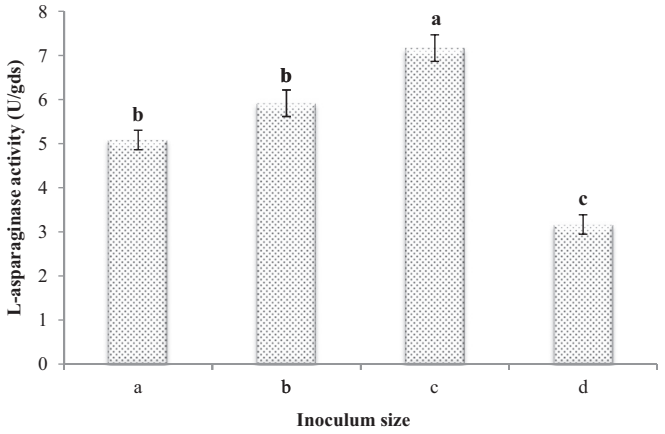

Fig. 4. Effect of inoculum size of substrate on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. (1 = 1 × 10^6, 2 = 1 × 10^7, 3 = 1 × 10^8 and 4 = 1 × 10^9 spores/gds). In each series, mean values represented with the same lower case alphabets (a–c) are not significantly different (at $P < 0.05$) according to DMRT.

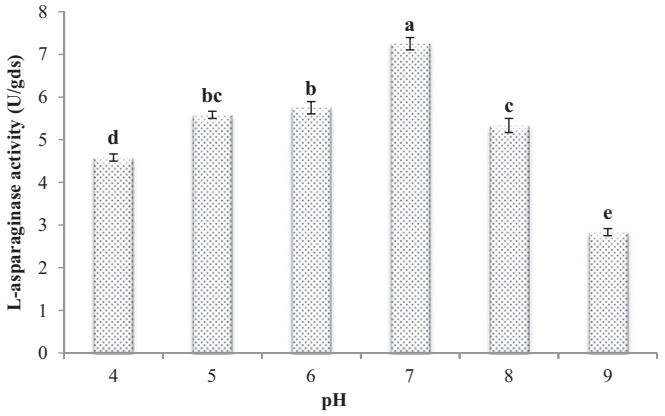

Fig. 5. Effect of initial pH on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. In each series, mean values represented with the same lower case alphabets (a–e) are not significantly different (at $P < 0.05$) according to DMRT.

pH for maximum production of L-asparaginase (7.33 U/gds) was observed when pH of the medium was adjusted to 7.0 (Fig. 5). Pallem et al. (2011) and Hosamani and Kaliwal (2011) also reported that an initial pH of 7.0 is most suited for L-asparaginase in *Fusarium oxysporum* and *Fusarium equiseti* when wheat bran and soybean meal was used as substrates. However, the enzyme activity decreased with increase in pH of the substrate beyond 7.0. This could be due to denaturation of enzymes by destruction of specific amino acid residues at higher pH. Swathi et al. (2014) on the contrary, reported initial pH of 9.0 is optimum for *Beauveria bassiana* (MSS 18/41) when wheat bran was used as substrate. Maximum L-asparaginase production by *Aspergillus terreus* KLS 2 was observed at pH 4.5 when carob pod was used as substrate (Siddalingeshwara et al., 2010). Thus, different fungal isolates have different pH optima for L-asparaginase production. This might be because fungal isolates respond distinctively to change in pH of the medium for enzyme production by providing ideal physiological pH for growth and development or by affecting the activity, structural stability and solubility of the enzyme.

L-asparaginase production gradually increased from 25 to 30 °C (Fig. 6) and maximum (7.66 U/gds) being at 30 °C. Further increase in temperature gradually decreased the enzyme production. This might be due to thermal denaturation of enzymes at higher temperature. In general, the temperature required for the production of enzymes in SSF is in the range of 25–32 °C (Lonsane et al., 1985). Our studies proved that optimum temperature for maximum production of L-asparaginase was 30 °C. Similar findings were reported from *Fusarium oxysporum* and

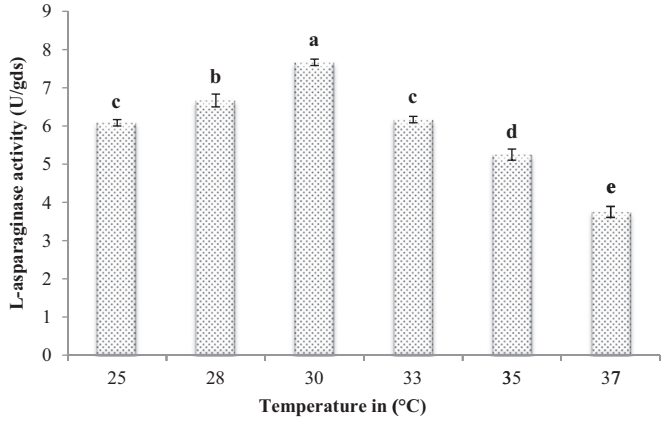

Fig. 6. Effect of temperature on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. In each series, mean values represented with the same lower case alphabets (a–e) are not significantly different (at $P < 0.05$) according to DMRT.

Cladosporium sp. by Pallem et al. (2011) and Kumar et al. (2013) respectively.

3.2.3. Effect of supplementation of carbon and nitrogen sources

The influence of supplementation of soybean meal with different carbon sources such as glucose, fructose, maltose, lactose, sucrose, starch, cellulose, xylose and ribose at 0.1% concentration on L-asparaginase production by *F. culmorum* (ASP-87) was studied. Addition of glucose resulted in maximum production of L-asparaginase (8.41 U/gds) followed by fructose (Fig. 7). Supplementation of substrate with xylose, ribose, sucrose and starch showed marginal increase in the production of L-asparaginase whereas, addition of maltose and cellulose resulted in decreased L-asparaginase production. Increase in the production of L-asparaginase with 0.1% glucose could be due to the fact that it is a simple and easily soluble sugar therefore, readily metabolized by fungi. It was also reported that glucose supported L-asparaginase production in other fungi such as *Aspergillus terreus* MTCC1782, *Fusarium oxysporum* and *Trichoderma viride* F2 (Gurunathan and Sahadevan, 2011; Pallem et al., 2011; Elshafei and El-Ghonemy, 2015). But on the other hand, glucose was also reported to be a repressor of L-asparaginase in *Enterobacter aerogenes* (Mukherjee et al., 2000). Further, to determine the optimum concentration of glucose as a supplement for L-asparaginase production, different concentrations (0.5%, 1.0%, 1.5%, 2.0% w/w) of glucose was carried out. But however, above 0.1% inhibited the enzyme synthesis (data not shown) and therefore, 0.1% (w/

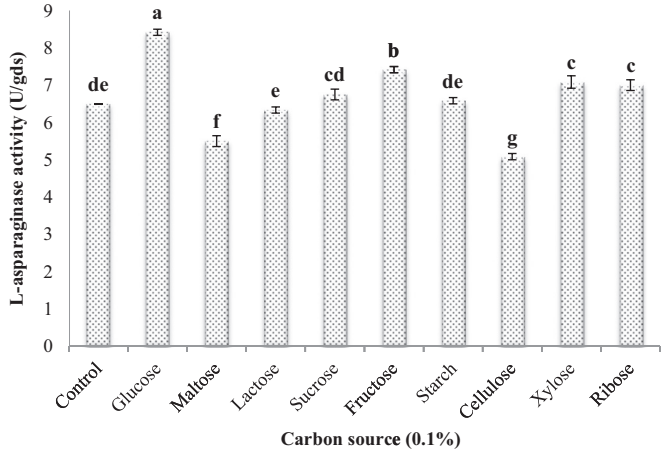

Fig. 7. Effect of supplementation of carbon sources on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. In each series, mean values represented with the same lower case alphabets (a–g) are not significantly different (at $P < 0.05$) according to DMRT.

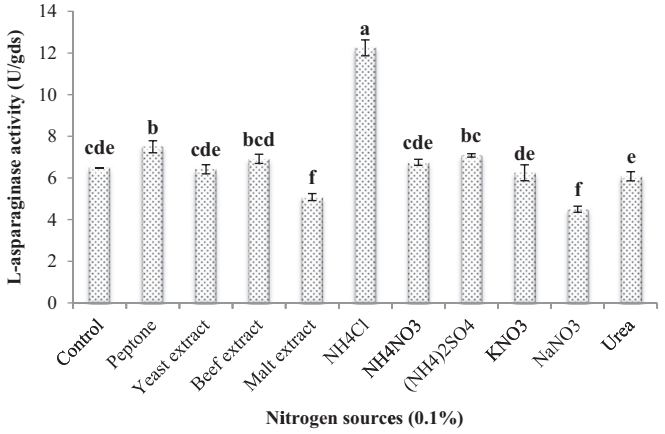

Fig. 8. Effect of supplementation of nitrogen sources on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. In each series, mean values represented with the same lower case alphabets (a–f) are not significantly different (at $P < 0.05$) according to DMRT.

w) of glucose is considered as the most optimum for L-asparaginase production in *Fusarium culmorum* (ASP-87).

The influence of supplementation of soybean meal with different organic and inorganic nitrogen sources such as peptone, yeast extract, beef extract, malt extract, ammonium chloride, ammonium nitrate, ammonium sulfate, potassium nitrate, sodium nitrate and urea at 0.1% concentration on L-asparaginase production by *F. culmorum* (ASP-87) was studied. Among all the nitrogen sources used, the maximum production of L-asparaginase 12.25 U/gds was observed when soybean meal was supplemented with 0.1% ammonium chloride followed by peptone with 7.5 U/gds activity (Fig. 8). Ammonium sulfate, ammonium nitrate and beef extract showed no significance in the production of L-asparaginase. However, addition of sodium nitrate and malt extract showed a negative effect resulting in decreased L-asparaginase production (Fig. 8). Pallem et al. (2011) and Elshafei and El-Ghonemy (2015) reported that malt extract and sodium nitrate enhanced the production of L-asparaginase in *Fusarium oxysporum* and *Trichoderma viride* F2 respectively. Hosamani and Kaliwal (2011) and Vijay and Raju (2014) reported ammonium sulfate enhanced the production of L-asparaginase in *Fusarium equiseti* and *Aspergillus terreus* MTCC 1782. Although soybean meal itself acts as nitrogen source in the growth medium, the influence of additional nitrogen source enhanced the production to 1.7 fold.

3.3. Effect of mixed substrate fermentation

Mixed solid substrates are viable substrates for SSF process (Benjamin and Pandey, 1998). Accordingly, a mixed SSF process was developed by mixing different substrates keeping soybean meal as constant. Interestingly, the mixture of soyabean meal with wheat bran increased the production of L-asparaginase (18.91 U/gds) in comparison to individual substrates alone followed by soybean meal with carob pod (12.33 U/gds) and soybean meal with fresh green peas (11.91 U/gds) (Table 2). However, mixture of other substrates with soybean meal did not have much influence on L-asparaginase production. There were very few work done previously on the production of L-asparaginase by mixed SSF. Vijay and Raju (2014) reported mixed substrates of pearl millet and finger millet yielded maximum production of L-asparaginase by *Aspergillus terreus* MTCC 1782. This is the first report on L-asparaginase production using a mixture of soybean meal and wheat bran (2.5 g and 2.5 g respectively). Further, the effect of different ratios (1:4, 2:3, 1:1, 3:2 and 4:1 w/w) of soybean meal and wheat bran was evaluated for L-asparaginase production. The results revealed maximum L-asparaginase production when soybean meal and wheat bran (1:1) were used in equal proportions (Fig. 9). Maximum yield of L-asparaginase

Table 2
Effect of mixed substrates on L-asparaginase production in *F. culmorum* (ASP- 87) under SSF.

Substrates	Enzyme activity (U/gds)
Soybean meal + Carob pod	12.33 ± 0.38
Soybean meal + Corn cob	8.91 ± 0.62
Soybean meal + Corn kernal	9.16 ± 0.14
Soybean meal + Gram husk	8.58 ± 0.38
Soybean meal + Green peas	11.91 ± 0.62
Soybean meal + Groundnut oil cake	10.91 ± 0.38
Soybean meal + Jowar husk	7.33 ± 0.14
Soybean meal + Mushroom powder	9.16 ± 0.38
Soybean meal + Soybean flour	10.66 ± 0.62
Soybean meal + Sugarcane bagassae	9.25 ± 0.5
Soyabean meal + Sunflower oil cake	10.33 ± 0.62
Soybean meal + Urad dhal waste	8.83 ± 0.38
Soybean meal + Wheat bran	18.91 ± 0.52

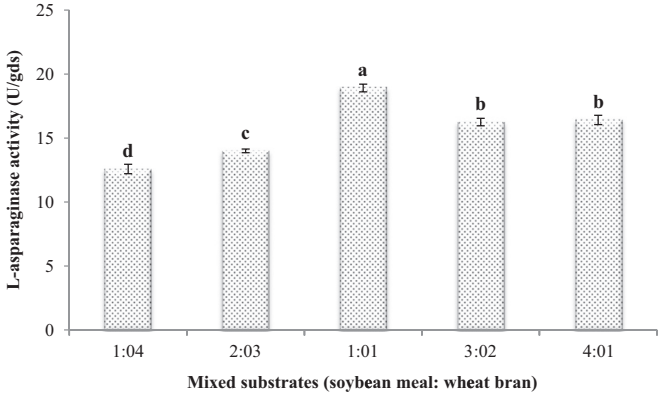

Fig. 9. Effect of mixed substrates on L-asparaginase production in *F. culmorum* (ASP-87) under SSF. In each series, mean values represented with the same lower case alphabets (a–d) are not significantly different (at $P < 0.05$) according to DMRT.

was obtained (24.5 U/gds) under optimized conditions. (data not shown). Soybean meal is the by-product of soybean processing industries, which act as a rich source of proteins and amino acids. Wheat bran is used as universal substrate as it contains sufficient nutrients and remains free under high moisture content, providing a large surface area for growth of microorganism. Both the substrates as such are rich in nutrients and therefore, act as a good substrate for the production of L-asparaginase.

4. Conclusion

In conclusion, utilization of agro based materials through biotechnological process is gaining more significance with the dual goal of waste disposal and better production. L-asparaginase, a biotechnologically important enzyme is commercially produced by submerged fermentation. Owing to its importance in pharmaceutical and food industries, cost effective technologies are needed for large scale production. Very few reports are available on screening and optimization of L-asparaginase production under SSF and this study is one of the few. Our present study has explored the use of several agro based substrates as sole source of nutrients for the production of L-asparaginase by *F. culmorum* (ASP-87) under SSF. Major parameters affecting the fermentative process for L-asparaginase production in SSF were studied and optimal levels were identified which could be exploited for large scale production. Mixed substrate fermentation using soybean meal and wheat bran showed promising results for L-asparaginase production in SSF. Although SmF is globally accepted method for the production of commercially important enzymes, we report that for L-asparaginase production SSF is the most suitable fermentation process which shows 8.2 fold higher enzyme production than in SmF

(Meghavarnam and Janakiraman, 2015).

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