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Production and optimization of L-asparaginase by *Bacillus* sp. KK2S4 from corn cob

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L-Asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an important enzyme which has anti-tumor properties. This paper describes the production and optimization of L-asparaginase by *Bacillus* sp. KK2S4 utilization of ground corn cob waste as substrate to reduce and manage the waste in our environment. Thirty-nine (39) bacterial isolates were screened for their L-asparaginase productivity and protein content. Only *Bacillus sp.* KK2S4 was identified before and selected as the most potent isolate. Modified M9 (MM9) minimal complete medium (glucose free) was used as production media for enzyme production. The highest enzyme production was detected at pH 5.0 in presence of lactose and $NaNO_3$ as the best carbon and nitrogen sources with high activity increasing percentage of 1058.9, 377.3 and 566.7%, respectively.

Key words: L-Asparaginase production, corn cob waste, enzyme optimization, anti-tumor agent.

INTRODUCTION

Many enzymes have been used as drugs, like wise L-asparaginase attracted much attention because of its use as effective therapeutic agent against lymphocytic leukemia and other kinds of cancer in man (Krasotkina et al., 2004). This enzyme is widely distributed, being found in animal, microbial and plant sources as well as large number of microorganisms that include *Erwinia carotovora*, *Pseudomonas stutzeri* (Mannan et al., 1995), *Pseudomonas aerugenosa* (Abdel-Fatteh and Olama, 2002) and *Escherichia coli* (Qin and Zhao, 2003; Jain et al., 2012). It has been observed that eukaryotic micro-organisms like yeast and fungi have a potential for asparaginase production (Pinheiro et al., 2001). L-Asparaginases (EC 3.5.1.1) hydrolyze L-asparagine to L-aspartate (also known as L-aspartic acid) and ammonia as shown in Figure 1 (Rani et al., 2012; Verma et al. 2007; Savitri and Azmi, 2003; Borek and Jaskólski, 2001). The effects of municipal solid waste compost and decomposed cow manure on L-asparaginase activities in submerged rice soil have been studied. L-Asparaginase and L-glutaminase activities were reported higher in decomposed cow manure, as compared to municipal solid waste compost treated soils due to higher amount of organic materials like water soluble organic carbon, carbohydrate and mineralized nitrogen in decomposed cow manure (Bhattacharya et al., 2007). This paper deals with the production and optimization of L-asparaginase from *Bacillus sp.* using corn cob waste as a substrate. There were no reports on production and optimization of L-asparaginase by using corn cob substrate.

MATERIALS AND METHODS

Isolation of bacterial isolates

Nutrient agar (NA) was prepared for bacterial isolation using serial dilution method from different sources(soil, water and food samples) collected from different areas of Kuantan, Pahang, Malaysia.

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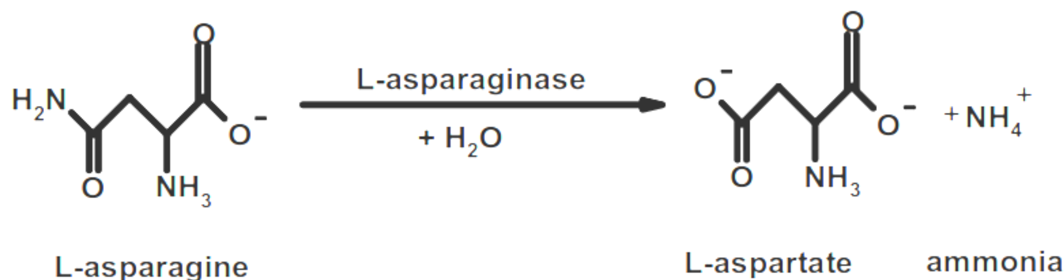

Figure 1. Schematic illustration of the reaction mechanism of L-asparaginase.

The plates were incubated at 37°C for 48 h. All isolates were purified and screened for L-asparaginase production when subjected to grow in the presence of corn cob waste.

Corn cob pretreatment and inoculum preparation

In this experiment, corn cob waste collected from any timber was used as a substrate for bacterial degradation power. Dried corn cob has been ground into fine pieces and treated with a sufficient amount of 4 N NaOH and kept at room temperature overnight. Then the corn cob was washed using tap water several times until the color of the water become clear or less dense. The pH was checked many times using pH meter until neutralize the solution then filtered using Muslin cloth to drain the water then left to dry in oven overnight at 40°C incubator. Bacterial suspension was prepared for inoculum purpose, about 50 ml nutrient broth media prepared into 100 ml conical flask and autoclaved for 20 min at 121°C, purified isolates were inoculated and incubated at 37°C for 48 h.

Production media preparation

Fifty (50) ml of modified M9 (MM9) minimal medium (2X) (glucose free) was prepared into 100 ml conical flask and 1.5 g of pre-treated corn cob was added and autoclaved at 121°C for 20 min and then inoculated with 5 ml of 24 h bacterial suspension and incubated at 37°C for 48 h. The control was run only in presence of MM9 inoculated with bacterial isolates (corn cob free).

Protein determination and enzyme assay

Cell-free filtrate was prepared (as crude enzyme obtained) after 48 h incubation; the production media were transferred into 15 ml conical centrifuge tube and then centrifuged at 4500 rpm for 6 min. Protein determination was carried out according to Lowry et al. (1951) method by Folin reaction and all samples in replicates and the absorbance was compared with standard curve which was prepared from bovine serum albumin (BSA). L-Asparaginase was assayed by Nessler's reaction according to Imada et al. (1973). This method is based on the amount of ammonia liberated from L-asparagine in the enzyme reaction using UV-visible spectrophotometer at wavelength of 450 nm. The reaction was initiated by adding 0.5 ml supernatant into 0.5 ml 0.04 M L-asparagine and 0.5 ml 0.5 M acetate buffer, pH 5.4, and incubated at 37°C for 30 min. After the incubation period the reaction was stopped by adding 0.5 ml of 1.5 M of trichloroacetic acid (TCA). 0.1 ml was taken from the above reaction mixture and added 3.75 ml distilled water and to that 0.2 ml Nessler's reagent was added and incubated for 20 min. The optical density (OD) was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA. The enzyme activity was expressed in International unit. One IU of L-asparaginase is defined as the amount of enzyme that liberates 1 µmol of ammonia per minute per ml [µmole/ml/min]. Standard curve of ammonium sulphate was used for calculating ammonia concentrations.

Parameters controlling enzyme productivity

The parameters controlling L-asparaginase production were carried out by only for the most potent bacterial isolate. Different four carbon and nitrogen sources (lactose, glucose, sucrose and starch) and ($NaNO_3$, yeast extract, KNO_3 and peptone) were used to optimize the enzyme production, respectively. Only 1.5 g of pre-treated corn cob substrate added to 49.5 ml of MM9 media into 100 ml conical flask, 0.5 ml of each source was added into the flasks and then inoculated with about 5 ml bacterial suspension of the selected most potent *Bacillus sp.* KK2S4 and incubated at 37°C for 48 h. On the other hand, different pH-values within the range of 4.0 to pH 9.0 were determined. At the end of incubation period, the cell-free filtrate was prepared for protein and enzyme assay.

RESULTS AND DISCUSSION

About 39 isolates were obtained and screened for both L-asparaginase production and protein content at 37°C for 48 h incubation period. Only four out of 39 isolates were selected and screened again according to higher enzyme productivity. *Bacillus sp.* KK2S4 identified was selected as the most potent isolate to continue for optimization (Table 1). Four different carbon and nitrogen sources were used and revealed that lactose exhibited maximum enzyme activity with (0.315±0.014 U/ml), specific activity (sp. act.) up to (0.157 U/mg) and the activity increased to 377.3% (Figure 2), while $NaNO_3$ showed that the maximum L-asparaginase activity up to (0.460±0.008 U/ml) and (0.216 U/mg) specific activity with increase of about 566.7% by *Bacillus sp.* KK2S4 isolate at 37°C for 48 h (Figure 3). On the other hand, pH 5.0 exhibited the maximum value for enzyme production at (0.649±0.001 U/ml) and specific activity of 0.504 U/mg with increase in activity to 1058.9% (Table 2) (Figure 4).

Lactose was indicated as the best carbon source in various studies in microbial production of L-asparaginase (Kenari et al., 2011; Savitri and Azmi, 2003). It is shown

Table 1. Selection of most potent isolate using treated corn cob waste at 37°C.

Isolate code	Asparaginase activity (450 nm)	Protein content (mg/ml)
	Mean ± SD	Mean ± SD
Control	0.098±0.010	0.290±0.007
KK2S4	0.261±0.014	0.569±0.000
KK2S5	0.239±0.005	0.590±0.004
PS1	0.233±0.005	0.494±0.008
CL14	0.185±0.005	0.533±0.003

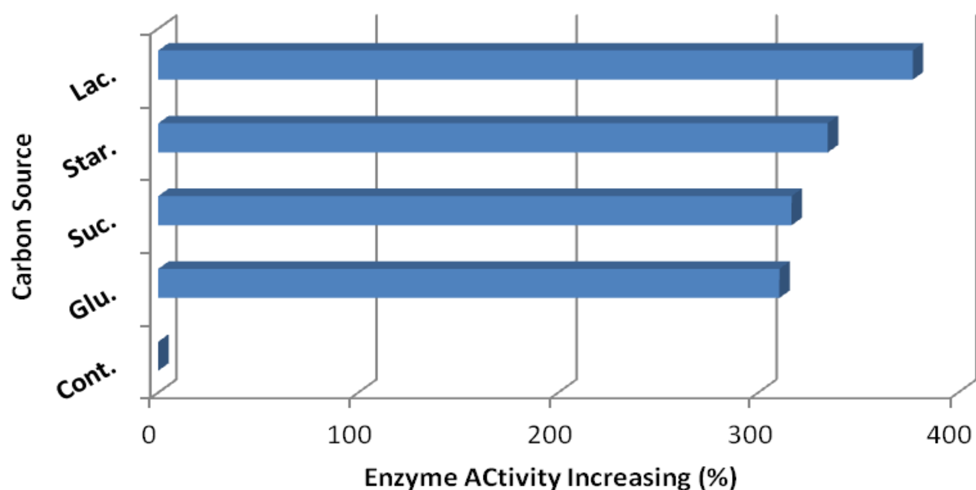

Figure 2. Graph of different carbon sources used showing activity increasing percentage.

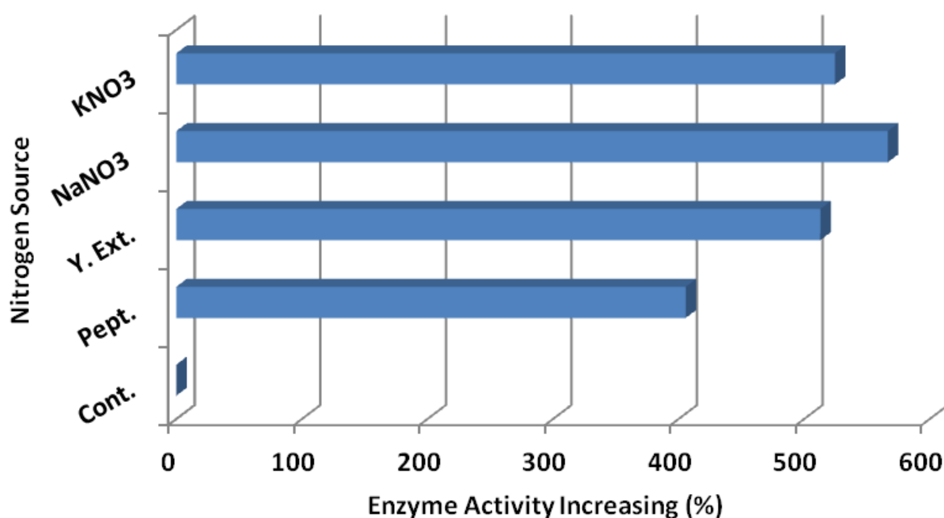

Figure 3. Graph of different nitrogen sources used showing activity increasing percentage.

that glucose and sucrose can inhibit the enzyme production; especially, glucose considered as a repressor for L-asparaginase production in bacteria may be due to some catabolites repression and inhibition of the components involved in lactate transport and lactate stimulated L-asparaginase synthesis (Kenari et al., 2011).

Table 2. Optimization of anti-cancer productivity from corn cob waste by KK2S4 at 37°C.

Parameter	Asparaginase activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Activity increasing (%)
	Mean ± SD	Mean ± SD		
Different carbon source				
Control	0.278±0.009	1.052±0.007	0.084	0.0
Glucose	0.271±0.006	1.782±0.043	0.152	310.6
Sucrose	0.275±0.009	1.777±0.028	0.155	316.7
Starch	0.287±0.002	1.812±0.004	0.158	334.8
Lactose	0.315±0.014	2.012±0.010	0.157	377.3
Different nitrogen sources				
Control	0.278±0.009	1.052±0.007	0.065	0.0
Peptone	0.349±0.009	3.190±0.019	0.109	405.8
Yeast extract	0.423±0.027	3.200±0.023	0.132	513.0
Sodium nitrate	0.460±0.008	2.132±0.052	0.216	566.7
Potassium nitrate	0.431±0.003	2.183±0.004	0.197	524.6
Different pH-values				
Control	0.452±0.006	0.955±0.002	0.059	0.0
4.0	0.599±0.015	1.001±0.027	0.598	969.6
5.0	0.649±0.001	1.288±0.018	0.504	1058.9
6.0	0.599±0.012	1.272±0.019	0.471	969.6
7.0	0.574±0.009	1.804±0.008	0.318	925.0
8.0	0.543±0.009	2.094±0.023	0.259	869.6
9.0	0.501±0.018	2.240±0.024	0.224	794.6

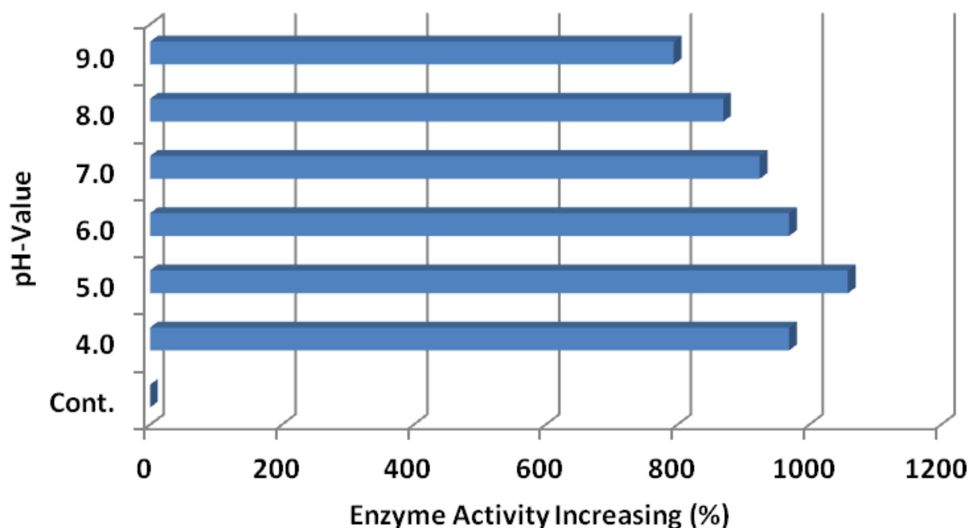

Figure 4. Graph of different pH-values showing activity increasing percentage.

The acidic nature of fermentation medium could inhibit L-asparaginase biosynthesis (Narayana et al., 2008). In a study using *Bacillus* strain (DKMBT10), the purified L-asparaginase has greater specific activity with glucose as carbon source (1.12 U/mg) when compared with maltose (1.05 U/mg) (Moorthy et al., 2010). In other reports that used *Bacillus cereus* MNTG-7, tapioca starch was found to be the best carbon source with positive coefficient

(6.390) (Sunitha et al., 2010). This could be due to the different strain of *Bacillus* sp. In the present study, nitrogen catabolite repression on enzyme formation was absent in *Bacillus sp.* With isolate, it was shown that all nitrogen sources gave better results than control. In contrast, most studies indicate that yeast extract is the best nitrogen source. According to Kenari et al. (2011), yeast extract showed the best nitrogen source for *E. coli* ATCC (11303) with 0.15 U/ml. Also found to be the most significant variable for *E. carotovora* MTCC 1428 (Deokar et al., 2010). Yeast extract is the best organic nitrogen source for *Fusarium equiseti* during L-asparaginase production under solid state fermentation (Hosamani and Kaliwal, 2011). *Streptomyces albidoflavus* also required yeast extract as nitrogen source for maximum L-asparaginase production (Narayana et al., 2008). In the present study, the result has proven that *Bacillus sp.* can produce maximum L-asparaginase enzyme at acidic environment. As the pH increases beyond pH 5.0, the L-asparaginase productivity also decreases consistently. This shows that alkaline condition is not suitable for *Bacillus sp.* When grown on corn cob as a substrate to yield optimal L-asparaginase enzyme. A study which has almost similar result was carried out using deseeded carob pod as substrate. L-Asparaginase produced by *Aspergillus terreus* KLS2 was found to be highest at pH 4.5 with L-asparaginase activity of 5.210 IU after 72 h of submerged fermentation process (Siddalingeshwara and Lingappa, 2010). For *S. albidoflavus*, as pH increases form pH 5.0, the L-asparaginase activity also increases. After pH 7.5, the L-asparaginase activity started to decreases (Narayana et al., 2008).

In conclusion, this is the first report of study that used *Bacillus sp.* and was selected to be the most potent microbial isolate for L-asparaginase (anti-cancer agent) production using corn cob as the sole carbon source at 37°C for 48 h incubation period. The enzyme was successfully enhanced and exhibited higher productivity at pH 5.0 in the presence of 20% lactose, sodium nitrate, 200 ppm asparagine and corn cob in the MM9 minimal complete medium as production media.

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