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Optimization of Cultural Conditions for the Biosynthesis of Phenylalanine ammonia lyase (PAL) by *Bacillus subtilis* GCB-31 on agro-industrial wastes

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Abstract

Phenylalanine ammonia lyase (PAL) is the key enzyme that catalyzes the conversion of L-Phenylalanine to ammonia and trans- cinnamic acid. In phenyl propanoid pathway, PAL is the first enzyme which involved in the biosynthesis of polyphenol compounds such as flavonoids and lignin in plants. The present study was conducted with the main objective to optimize the phenylalanine ammonia lyase (PAL) production process by *Bacillus subtilis* GCB-31 on agro-industrial wastes as carrier substrates in solid state fermentation. The maximum PAL activity was obtained at 30 ^{o}C after 96 h using 1 mL inoculum level. 10g of mixed substrates (Corn Stover+ Corn Cobs+ Banana stalk) was optimized for maximum L-asparaginase production. Agro-industrial wastes, nitrogen sources and carbon sources were also optimized for the maximum production of enzyme (58.77±2.94 U/mL). It was also found that glucose and ammonium sulphate as the best additional carbon and nitrogen sources respectively. It can be inferred from this study that optimization of fermentation process enhanced the production of desired enzyme.

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Introduction

Phenylalanine ammonia lyase (EC 4.3.1.5) catalyzes the catabolism of phenylalanine to trans cinnamate and NH_3. Moreover, PAL has also been used for the conversion of trans-cinnamate to L-phenylalanine. This enzyme is widely distributed in higher plants, some yeasts, cyanabacteria, Streptomyces and fungi, yeasts, (Moffitt *et al.,* 2007; Cui *et al*., 2014). PAL has greatly importance for catabolic function in yeast and microorganisms. Moreover, the PAL enzyme has been involved in the biosynthesis of antibiotic and photoactive yellow (Berner *et al.,* 2006).

In higher plants, PAL has significant role in plant development and its response to environmental stimuli (Santiago *et al.,* 2009). PAL has also been reported in microorganisms, fungi and few bacteria (Ogata *et al*., 1966; D'Cunha *et al.,* 1996; Berner *et al*., 2006; Chesters *et al.,* 2012; Zhu *et al*., 2012). PAL has many clinical and industrial applications such as the treatment of phenylketonuria, quantitative analysis of serum Phenyl alanine, production of L-phenylalanine, and in the formulation of low L-phenylalanine containing diets. However, the scientist facing the problem due to instability of PAL during application relatively low specific activity. Recently, biotechnological tools have been using to improve PAL activity and stability for various purposes.

Therefore, the aim of present project was to study the production of therapeutically and industrially important phenylalanine ammonia lyase and optimization of cultural conditions for maximum production of PAL by *Bacillus subtils* GCB-31 using agro industrial wastes.

Materials and methods

Microorganism and carrier substrates

The pure local culture strain of *B. subtilis* GCB-31 mutant strain (stock culture) was maintained in a refrigerator (4°C) on nutrient agar medium (Oxoid, Hampshire, UK). Different agricultural by products used in the present study such as Corn cobs, Corn stover, wheat straw, Banana stalk and Baggase were obtained from the local market.

Mutagenesis by gamma irradiation

Seven tubes each 10 mL of spore suspension was irradiated with γ-rays in a Cz137 source at different ranges 1-4KGy (1, 1.5, 2, 2.5, 3, 3.5 and 4) for induction of mutation (Iftikhar *et al*., 2010). The irradiated spores were diluted serially with nutrient broth medium and then an appropriate volume was poured on nutrient agar containing plates. 0.1 % Triton X-100 was used to inhibit the growth of other bacterial colonies in the nutrient agar medium. It was shown clear zone after the incubation (30 °C) of plates in the dark room for 48 hours. The strains which produced larger zone were scratched, dissolved and homogenized in inoculums and activity of the phenylalanine ammonia lyase was determined using spectrophotometer.

Solid state fermentation technique

Solid state fermentation technique was used for production of bacterial PAL. Ten grams of substrate was moistened with 7mL of nutrient broth in 250 mL conical flask. These flasks were sterilized at 121°C for 15 minutes. After cooling to room temperature, all flasks were inoculated with 1ml of homogenous spore suspension was aseptically transferred to each cotton wool plugged conical flask and the flasks were incubated for 48 h in orbital shaker. The ingredients of the flask were prepared for enzyme extraction and filtrate was used for estimation of phenylalanine ammonia lyase activity (Javed *et al.,* 2007).

Analytical

PAL activity was analyzed as the rate of conversion of L-phenylalanine into trans-cinnamic acid [(E)-cinnamic acid] at 270nm in a UV-Vis spectrophotometer.19 Samples containing 0.1 ml of enzyme extract were treated with 0.5 ml of 0.1M trisodium borate buffer (pH 8.5) and 0.5 ml of 12 mM L-phenylalanine in the same buffer. The volume of reaction mixture was made up to 3ml with deionized H_2O and kept for incubation at 30°C for 30 minutes. Immediately mixed by inversion and recorded the increase in absorbance at 270nm for approximately 5 minutes. The total protein contents of the samples were determined by the method of (Lowry *et al.,* 1951)

using bovine serum albumin (BSA) as a reference standard.

Statistical analysis

Comparison of means were done using Duncan multiple range test. Statistical significance of the differences between mean values was assessed two way analysis of ANOVA, using Minitab 2000 version 13.2 statistical software (Steel *et al.,* 1997).

Result and discussion

Five different substrates such as corn cobs, corn stover, wheat straw, banana stalk and bagasse were tested for the production of phenylalanine ammonia lyase by *Bacillus subtilis* GCB-31 (Table 1).

Table 1. Selection of substrate for PAL production by *Bacillus subtilis* GCB-31.

Sr. No.	Substrate	Asparaginase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)
1	Corn stover	9.11±0.76	0.214±0.02	31.14
2	Corn Cobs	8.42±1.01	0.198±0.04	27.31
3	Banana Stalk	6.23±1.18	0.257±0.03	26.76
4	Wheat Straw	11.92±1.13	0.321±0.021	37.88
5	Bagasse	7.32±0.69	0.184±0.037	35.24

Values (mean± SD, $p \leq 0.05$).

Table 2. Selection of bi-substrate forPAL production by *Bacillus subtilis* GCB-31.

Sr. No.	Combination of Substrate (1:1)	Asparaginase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)
1	S1(Corn Stover+Corn Cobs)	21.64±2.66	0.22±0.02	94.14
2	S2(Corn Stover+Banana Stalk)	27.46±2.39	0.31±0.011	103.71
3	S3(Corn Stover+Wheat Straw)	31.77±1.66	0.28±0.017	214
4	S4(Corn Stover+Baggase)	37.36±1.31	0.33±0.021	186.26
5	S5(Corn Cob+Banana Stalk)	34.28±1.72	0.25±0.026	207.63
6	S6(Corn Cob+Wheat Straw)	33.54±1.27	0.30±0.019	318.14
7	S7(Corn Cob+Baggase)	29.87±2.39	0.27±0.023	257.31
8	S8(Wheat Straw+Banana Stalk)	48.33±1.61	0.23±0.01	268.64
9	S9(Banana Stalk+Baggase)	35.62±1.79	0.35±0.013	125.17
10	S10(Wheat Straw+Baggase)	25.15±2.45	0.26±0.014	229.34

Values (mean± SD, $p \leq 0.05$.

Wheat straw showed significantly highest enzyme activity (11.92±1.13) as compared to other substrate while minimum Phenylalanine ammonia lyase was obtained (6.23±1.18) using corn cob. Therefore, wheat straw was observed as best carbon and nitrogen source among all other agro industrial substrates. However, it might be possible that rest of all substrates not fulfilled the nutritional requirement of the organism. Varalakhshmi and Raju, 2013 reported bajra seed flour has found to be good carrier substrate for L-asparaginase production. This work is not in accordance with above reported work due to difference in utilized substrates and prevailed higher results of different enzyme activities.

Five carrier substrates such as S1, S2, S3, S4, S5, S6, S7, S8, S9 and S10 were checked for the production of Phenylalanine ammonia lyase by *Bacillus subtilis* GCB-31 (Table 2). It was found that S8 (Wheat Straw; Banana Stalk) gave significantly higher enzyme activity (48.33±1.61U/mL) than other substrates. Moreover, S1 (Corn Stover; Corn Cobs) showed

minimum production of phenylalanine ammonia lyase (21.64±2.66 U/mL). Combination S8 has given maximum PAL activity with respect to growth of microorganism. Thus, the S8 (Wheat straw; banana stalk) combination was selected for further studies because this combination attained the nutritional demands of the bacteria. Edwinoliver *et al.,* 2013 obtained a 3-fold increase in activity using tri substrate while Iftikhar *et al.,* 2011 found 8 folds increase than single substrate fermentation.

Table 3. Effect of substrate to diluent ratioon production of PAL by *Bacillus subtilis* GCB-31.

Sr. No.	Substrate to diluent ratio		Asparaginase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)
	Substrate (g)	Diluent (mL)			
1	10	8.0	49.64±1.62	0.34±0.014	205.4
2	10	16.0	38.41±1.87	0.41±0.015	194.21
3	10	24.0	39.51±1.94	0.51±0.029	315.62
4	10	32.0	26.33±2.45	0.36±0.013	256.10

Values (mean± SD, $p \leq 0.05$).

Table 4. Effect of incubation time on production ofPAL by *Bacillus subtilis* GCB-31.

Sr. No.	Incubation time (h)	Asparaginase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)
1	24	38.14±2.25	0.23±0.012	218.15
2	48	42.74±1.19	0.49±0.01	309.54
3	72	50.33±1.83	0.65±0.023	322.41
4	96	44.36±2.62	0.74±0.025	276.64
5	120	35.22±1.52	0.61±0.014	260.21

Values (mean± SD, $p \leq 0.05$).

The substrate to diluent ratio has great impact on the secondary metabolites production under solid state fermentation. Thus phenylalanine ammonia lyase by *Bacillus subtilis*GCB-31was carried out. Various volumes 8-32mL od diluents were investigated (Table 3). The maximum (49.64±1.62 U/mL) production of phenyl ammonia lyase was obtained by using 8 mL and minimum PAL activity (26.33±2.45U/mL) was attained at 1:4 substrates to diluent ratio.

Table 5. Effect of incubation temperature on production ofPAL by *Bacillus subtilis* GCB-31.

Sr. No.	Temperature (°C)	Asparaginase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)
1	20	43.30±1.43	0.23±0.012	218.15
2	25	45.21±2.11	0.49±0.01	309.54
3	30	51.52±1.55	0.65±0.023	322.41
4	35	40.28±1.31	0.74±0.025	276.64
5	40	33.46±2.44	0.61±0.014	260.21

Values (mean± SD, $p \leq 0.05$).

These findings indicated that at very higher moisture contents, the phenyl ammonia lyase production was significantly decreased because of lesser porosity followed by decrease in exchange of gases resulting in low enzyme production according to Silman *et al.*, 1979. At lower moisture contents, PAL activity was

found to be very low due to the lower solubility nutrients and degree of swelling of the substrate created higher water tension Panda *et al.,* 2016 has reported 70% moisture content to be optimum for working on the production of fungal derived enzymes. Maximum production of phenylalanine ammonia lyase (50.33±1.83 U/mL) obtained after 96 hours of inoculation (Table 4). Thus, for further studies 96h incubation period was optimized. Above and below 96 h time interval, comparatively lower phenylalanine ammonia lyase production was obtained. Depletion of nutrients or loss of moisture can cause enzyme inactivation. Cui, 2010 observed that under optimized culture conditions, the maximum recombinant PAL activity was obtained at 18 h. However, under original culture conditions, the maximum recombinant PAL activity was obtained at 22 h.

Table 6. Effect of inoculum size on production ofPAL by *Bacillus subtilis* GCB-31.

Sr. No.	Inoculum size (mL)	Asparaginase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)
1	1.0	54.14±2.65	0.84±0.04	222.54
2	2.0	51.37±2.34	0.29±0.021	291.25
3	3.0	48.28±1.48	0.47±0.037	167.46
4	4.0	35.44±1.67	0.34±0.074	354.19
5	5.0	32.44±2.73	0.65±0.09	387.35

Values (mean± SD, $p \leq 0.05$).

Incubation temperature has profound effect on the production of phenylalanine ammonia lyase by *Bacillus subtilis* GCB-31in solid state fermentation. Various incubation temperatures such as 20°C, 25°C, 30°C, 35°C and 40°Cwere tested for PAL production (Table 5). Maximum (51.52±1.55 U/mL) PAL activity was attained at 30°C and below or above optimum temperature could not favor to achieve the maximum enzymatic activity because of the denaturation of enzyme at higher temperatures. Our results are best supported by Dutta and Banerjee, 2004 work. Mukhtar and Haq, 2008 obtained 37°C optimum temperatures for *Bacillus subtilis* IH-72 $^{EMS-8}$ alkaline protease.

Table 7. Effect of different carbon sources on production ofPAL by *Bacillus subtilis* GCB-31.

Sr. No.	Carbon sources	Asparaginase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)
1	Glucose	56.21±1.36	0.79±0.031	356.22
2	Sucrose	41.55±2.68	0.84±0.023	288.64
3	Starch	29.54±1.83	0.56±0.037	207.57
4	Maltose	49.65±2.64	0.66±0.051	265.39
5	Fructose	35.15±1.60	0.89±0.013	347.35

Values (mean± SD, $p \leq 0.05$).

The effect of Inoculum size on the production of value added secondary metabolites by solid state fermentation have been studied. The various inoculum levels (1.0-5.0 mL) for production of phenylalanine ammonia lyase have been shown in Table 6. Maximum PAL activity (54.14±2.65 U/mL) was found at 1 mL inoculum and it has been selected for further study. Initially asparaginase production increased by inoculum size but it gradually decreased by increase in inoculum size. Varalakhshmi and Raju, 2013 also optimized 1.0 mL of inoculum for maximum L-asparaginase production. However,

Nadeem *et al.,* 2007 found maximum enzyme activity with *Bacillus strain* at 2% inoculum size.

The various carbon sources such as fructose, glucose, maltose, sucrose and starch have been used to find out their effect on the production of phenylalanine ammonia lyase by *Bacillus subtilis*GCB-31(Table 7). The maximum growth of organism and production of PAL (56.21±1.36 U/mL) was achieved on glucose supplementation to the fermentation medium as compared to rest of carbon sources. Rest of all carbon sources has shown little or no effect on PAL production, which might be due to the contamination problems. Our work is in accordance with the work of (Zhang and Cui, 2012).

Table 8. Effect of different nitrogen sources on production ofPAL by *Bacillus subtilis* GCB-31.

Sr. No.	Nitrogen sources	Asparaginase activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)
1	$NaNO_3$	43.87±1.84	0.87±0.025	354.1
2	NH_4Cl	47.64±1.33	0.73±0.046	269.25
3	Urea	37.65±2.91	0.65±0.07	385.12
4	Peptone	51.61±1.61	0.37±0.03	275.35
5	Yeast Extract	45.21±2.77	0.68±0.051	277.50
6	Ammonium sulphate	58.77±2.94	0.79±0.05	175.63

Values (mean± SD, $p \leq 0.05$).

The effect of different nitrogen sources such as $NaNO_3$, ammonium sulphate, yeast extract, urea, NH_4Cl and peptone on the production of PAL by *Baciilus subtilis* GCB-31(Table 8). According to the results, on addition of ammonium sulphate to the medium, the maximum PAL activity (58.77±2.94 U/mL) was obtained. Thus, ammonium sulphate found as best nitrogen source to achieve highest production of PAL. This result is in accordance with Cui, 2010 who reported ammonium phosphate (7.02g/L) has given maximum production of PAL. Moreover, Nagger, 2015 have also found ammonium sulphate as a best source of nitrogen to support the growth of microorganism.

Conclusion

It can be concluded from the above study that optimization of fermentation process has significantly enhanced the phenylalanine ammonia lyase production by *Bacillus subtilis* GCB-31 strain.

Refrences

Baskar G, Renganathan S. 2011. Design of experiments and ANN linked genetic algorithm for modeling and optimization of l-asparaginase production by Aspergillus terreus MTCC 1782. Biotechnology and Bioprocess Engineering **16**, 50-8. http://doi.org/10.1007/s12257-010-0119-7

Berner M, Krug D, Bihlmaier C, Vente A, Müller R, Bechthold A. 2006. Genes and enzymes involved in caffeic acid biosynthesis in the actinomycete Saccharothrixespanaensis. Journal of bacteriology **188,** 2666-73. http://doi.org/10.1128/JB.188.7.2666-2673.2006

Chesters C, Wilding M, Goodall M, Micklefield J. 2012. Thermal bifunctionality of bacterial phenylalanine aminomutase and ammonia lyase enzymes. Angewandte Chemie International Edition **51,** 4344-8. https://doi.org/10.1002/anie.201200669

Cui JD, Qiu JQ, Fan XW, Jia SR, Tan ZL. 2014. Biotechnological production and applications of microbial phenylalanine ammonia lyase: a recent review. Critical Reviews in Biotechnology **34,** 258-68. https://doi.org/10.3109/07388551.2013.791660

D'Cunha GB, Satyanarayan V, Nair PM. 1996. Stabilization of phenylalanine ammonia lyase containing *Rhodotorulaglutinis* cells for the

continuous synthesis of L-phenylalanine methyl ester/96. Enzyme and Microbial Technology **19**, 421-7.
https://doi.org/10.1016/S0141-0229(96)00013-0

Dutta JR, Dutta PK, Banerjee R. 2004. Optimization of culture parameters for extracellular protease production from a newly isolated Pseudomonas sp. using response surface and artificial neural network models. Process Biochemistry **39,** 2193-8.
http://doi.org/10.1007/s13765-014-4194-x

E-A El-Naggar N. 2012. Extracellular production of the oncolytic enzyme, L-asparaginase, by newly isolated *Streptomyces sp.* strain NEAE-95 as potential microbial cell factories: Optimization of culture conditions using response surface methodology. Current PharmaceuticalBiotechnology **16,** 162-78.
https://doi.org/10.2174/1389201015666141113123910

Edwinoliver NG, Thirunavukarasu K, Naidu RB, Gowthaman MK, Kambe TN, Kamini NR. 2010. Scale up of a novel tri-substrate fermentation for enhanced production of *Aspergillus niger* lipase for tallow hydrolysis. Bioresource Technology **101,** 6791-6.
https://doi.org/10.1016/j.biortech.2010.03.091

Iftikhar T, Niaz M, Nisa Z, Tariq AS, Khalid MN, Jabeen R. 2011. Optimization of cultural conditions for the biosynthesis of lipases by *Penicillium chrysogenum* (MBL 22) through solid state fermentation. Pakistan Journal of Botany **43,** 2201-6.

Javed S, Meraj M, Mahmood S, Hameed A, Naz F, Hassan S, Irfan R. 2017. Biosynthesis of lovastatin using agro-industrial wastes as carrier substrates. Tropical Journal of Pharmaceutical Research **16,** 263-9.
http://dx.doi.org/10.4314/tjpr.v16i2.

Kong JQ. 2015. Phenylalanine ammonia-lyase, a key component used for phenylpropanoids production by metabolic engineering. RSC Advances **5,** 62587-603.
http://doi.org/10.1039/C5RA08196C

Lowry OH, Roserbrough N, Farr AL, Randall R 1951. Protein measurement with folin phenol reagent. Journal of Biological Chemistry **193,** 265-275.

Moffitt MC, Louie GV, Bowman ME, Pence J, Noel JP, Moore BS. 2007. Discovery of two cyanobacterial phenylalanine ammonia lyases: kinetic and structural characterization. Biochemistry **46,** 1004-12.
https://doi.org/10.1021/bi061774g

Mukhtar H, Haq I. 2008. Production of alkaline protease by *Bacillus subtilis* and its application as a depilating agent in leather processing. Pakistan Journal of Botany **40,** 1673-9.

Nadeem M, Qazi JI, Baig S, Syed QU. 2007. Studies on commercially important alkaline protease from *Bacillus Lichniformis* N-2 isolated from decaying organic soil. Turkish Journal of Biochemistry-turkbiyokimyadergisi **32,** 171-7.
http://doi.org/10.4172/1948-5948.1000103

Panda SK, Mishra SS, Kayitesi E, Ray RC. 2016. Microbial-processing of fruit and vegetable wastes for production of vital enzymes and organic acids: Biotechnology and scopes. Environmental research **1,** 161-72.
https://doi.org/10.1016/j.envres.2015.12.035

Santiago R, De Armas R, Legaz ME, Vicente C. 2009. Changes in phenolic acids content, phenylalanine ammonia-lyase and peroxidase activities in sugarcane leaves induced by elicitors isolated from Xanthomonasalbilineans. Australasian Plant Pathology **38,** 357-65.
http://doi.org/10.1071/AP09090815319109/040357

Silman RW, Conway HF, Anderson RA, Bagley EB. Production of aflatoxin in corn by a large-scale Solid-substrate fermentation process. Biotechnology and Bioengineering **21,** 1799-808. http://dx.doi.org/10.1002/bit.260211008

Steel RG, Torrie JH, Dickey DA. 1979. Principles and procedures of statistics. Pages: 400-428. A biometrical approach **3.** https://doi.org/10.1002/bimj.19620040313

Varalakshmi V, Raju KJ. 2013. Optimization of l-asparaginase production *by Aspergillus terreus* MTCC 1782 using bajra seed flour under solid state fermentation. International Journal of Resource Engineering and Technology **2,** 121-9. https://doi.org/10.15623/ijret.2013.0209020

Zhu YX, Liao SY, Ye J, Zhang H. 2012. Cloning and characterization of a novel tyrosine ammonia lyase-encoding gene involved in bagremycins biosynthesis in *Streptomyces sp.* Biotechnology Letters **34,** 269–74. https://doi.org/10.1007/s10529-011-0755-9