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OPTIMIZATION OF AMINO ACID PRODUCTION FROM CHICKEN FEATHER BY *BACILLUS AMYLOLIQUEFACIENS* USING RESPONSE SURFACE METHODOLOGY AS A NEW TOOL FOR POWDERY MILDEW DISEASE BIOCONTROL

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ABSTRACT

The present work aimed to optimize amino acids production by *Bacillus amyloliquefaciens* 35s from chicken feather using response surface methodology (RSM) and applying the produced amino acids in biological control of powdery mildew disease. Variables affecting amino acids production from B. *amyloliquefaciens* 35s were screened using Plackett Burman design. Factors showed to be significant to produce amino acids were optimized using central composite design (CCD) of RSM at three coded levels (-1, 0, +1). All obtained data were analyzed by ANOVA with post hoc multiple comparison analysis performed using Tukey's HSD. TGY (tryptone, glucose, yeast extract) was the basal medium. Impacts of medium components were studied using Plackett-Burman design. "Design Expert 12" State-Ease was used to analyze the experimental Plackett- Burman design. Feather, yeast extract, $CaCO_3$, KH_2PO_4 and agitation rate (using shake flask) were optimized statistically by the CCD design of the RSM. Validation of statistical model of the medium components was done by carrying out the experiment at optimum conditions of the process parameters as determined by the model. Amino acids types were identified by amino acid analyzer. Among the significant medium components, feather, yeast extract, $CaCO_3$, KH_2PO_4 and agitation rate showed to have significant effect on amino acids production. Predicted maximum amino acids production was observed near the mid-point concentrations of the mentioned above factors and the experimental value (6.55 g/l) was very close to the predicted value of (6.60 g/l) predicted by the model. Amino acid analyzer analysis for the produced amino acids shows the release of asparagine, threonine, serine, glutamine, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenyl alanine, histidine, lysine and arginine, respectively. Effect of chicken feather hydrolysate on cucumber powdery mildew disease was tested by applying foliar application with different concentrations (0, 5, 10, 15, and 20% v/v), then degree of infection and sporulation on cucumber plants were assessed. The lowest concentration of chicken feather hydrolysate showed the lowest effect on disease index. Conversely, cucumber resistance increased by the increasing of feather's hydrolysate concentrations associated with a clear reduction in the disease index and sporulation. Aside from, extra foliar application showed a decrease in the disease index for all concentrations compared with untreated plants. Foliar application with 20% concentration of chicken feather hydrolysate had the highest stimulation of the studied oxidative enzymes *i.e.,* peroxidase (POX), phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO) and superoxide dismutase (SOD).

Keywords: Chicken feather hydrolysate; *Bacillus amyloliquefaciens* 35s; Amino acids; RSM; Biocontrol; Powdery mildew; Cucumber; Oxidative enzymes.

INTRODUCTION

Nine million tons of feathers are produced each year all around the world as a cheap by-product during the poultry processing industry. It composed of about 90% protein as keratin, one of the fibrous and insoluble structural proteins, due to the presence of hydrogen, cysteine di-sulphide bonds, and hydrophobic interlinkages. Acid hydrolysis of keratin using trypsin, papain and pepsin is difficult because of its high mechanical strength and its slow degradation process in nature. Hydrolyzing feathers by the traditional physical and chemical methods conducts a big loss in its amino acids content as lysine and methionine. Contrarily, feathers microbial biodegradation is an alternative tool for keratin wastes hydrolyzing. So, biodegradable feathers using bacteria such as *Bacillus* spp **(Tamreihao et al 2017)** are considered one of the rich sources of valuable proteins and essential amino acids (D'Este et al 2018). In fact, not only, hazardous chemical processes can be replaced by microbial hydrolysis of keratin. **(Box and Wilson, 1951)** but also, it provides a cheap and a mild reaction conditions to produce essential amino acids **(Chauhan and Gupta, 2004)** as it is applied as a biocontrol agent against plant pathogens **(Joo and Chang, 2006)**.

Powdery mildew is a disease infects greenhouse and field grown cucumber plants widely **(Rankovic, 2003; Cosme et al 2012)**. The common pathogens of this disease are *Podosphaera xanthii* (syn *Podosphaera fuliginea*) and *Golovinomyces cichoracearum* (syn *Erysiphe cichoracearum*). **Zitter et al (1996)** affirmed that growth and pathogenicity *of P. xanthii* increased rapidly under greenhouse conditions. Powdery mildew symptoms form white powder growth -talcum like- on leaf surfaces and stems of cucumber plants. As proved by **(McGrath 1996; Dik et al 2004)**, fungal growth on plant surfaces not only disrupt photosynthesis and respiration processes leading to leaves fade and death but also, reduce both of quantitative and qualitative cucumber yield. Chemical control is commonly used tool for controlling the disease. However, it is not an efficient way due to the control of *P. xanthii* with fungicides **(Reuveni et al 1996)**. Moreover, chemicals are not eco-friendly causing harmful effects for ecosystems. Therefore, it is needed indeed to find alternative eco- friendly tools to control the disease. Recently, many researchers have provided the effective role of amino acids on controlling of plant pathogens. According to **(Zheng et al 2011)**, increasing enzymatic activities of polyphenol oxidase,phenylalanine ammonia-lyase and chitinase, β-1,3-glucanase leads to reduce the tomato fruit lesion size by *Botrytis cinerea* and activates plant defense when spraying the green mature tomato fruits using different levels of arginine as 0.5, 1, and 5 mM, respectively. Also, **Bahadur et al (2012)** found that applying foliar spraying on pea leaves by different concentrations of L-phenylalanine (50, 100 and 150 ppm) resulted in increasing the PAL activity as well as reduction in the spore germination of the pathogenic *Erysiphe pisi*. In the same manner, **Hasabi et al (2014)** showed that foliar spraying of L- methionine on lime leaves resulted in a significant reduction of canker lesions as well as significantly increasing of the plant induced resistance by the way of increasing the activities of both phenylalanine ammonia-lyase and peroxidase enzymes compared to control treatments. Using small doses of amino acids (glutamate, cysteine, phenylalanine, and glycine) by foliar application or seed treatment led to increase the activity of antioxidant enzymes (phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase) in soybean plants **(Teixeira et al 2017)**. Therefore, the aim of the current research was to identify significant variables affecting amino acids production during biodegrading of feathers by *B. amyloliquefaciens*. Then, evaluating the recycling of chicken feathers as an effective and low cost alternative controlling powdery mildew diseases in cucumber.

MATERIALS AND METHODS

Microorganism

B. amyloliquefaciens 35s strain was previously isolated from feather waste in Cairo, Egypt and identified **(Nassar et al 2015)**. For culture maintenance, nutrient agar slants were inoculated with loopful of the strain and maintained at 4°C. Subculturing was done every month. Nutrient agar composed of (g/l): beef extract 3, peptone 5, agar 15.

Hydrolysis of chicken feather

Chicken feathers were obtained by their collection from local poultry slaughterhouse in Cairo, Egypt. Obtaining of chicken feather hydrolysate was performed as described by **(Nassar et al 2015)**. Prepared chicken feather meal powder was maintained at room temperature for further studies.

Standard inoculum

Standard inoculum was prepared by inoculating 50 ml of TGY broth medium composed of (g/l): tryptone (10), glucose (10), yeast extract (5) in 250 ml conical flasks with a loop of tested culture. The inoculated flask was incubated on a rotary shaking incubator (Lab-line Inc. Ltd.) at the rate of 120 rpm for 24 h at 37°C and considered as the standard inoculum (1 ml contained 6.0 – 7.0 x 105 viable cells) for shake flasks.

Plackett-Burman design (PBD) for screening of the growth culture main parameters

PBD was used to screen the main significant medium components according to their main effects using a 12 trial Plackett- Burman experiments through one-way ANOVA by "Design Expert® 12.0" Stat-Ease, Inc., Minneapolis, USA, ten ingredients were selected for the study at two factor levels, high (+1) and low (-1) **Table 1**. Feather, glucose, yeast extract, peptone, $CaCO_3$, KH_2PO_4, temperature, pH, inoculum size agitation speed and incubation time. **Table 2** records the factor name, symbol code, and coded levels of the screened variable parameters. Main media components and their levels used in the experimental design using the symbol code and the actual level of the variables. All flasks were inoculated and incubated at 37˚C at 150 rpm for 24 h. for performing the total amino acid assay.

Central Composite Design (CCD) design for optimization of media components levels

According to the results obtained from placket Burman design, five parameters (feather, yeast extract, $CaCO_3$, KH_2PO_4 and agitation rate) were selected as the main significant factors for amino acids production. Optimization was done using a 31 full factorial CCD design. **Tables 3 and 4** showed both of the minimum and maximum levels of parameters tested according to their actual and coded values. Concerning the achievement of experiments, the maximum average of amino acids production was studied as the dependent variable parameter or response (Y). Multiple regression method was calculated to fit the second order polynomial equation in the recorded data. The model equation for this five-factor system was:

AA =-142.21571+ 12.98718feather+ 44.24693yeast extract+5.56997 $CaCO_3$+78.35570 KH_2PO_4 + 0.786464 agitation +0.064500 feather * yeast extract-0.040571 feather * $CaCO_3$ -0.465000 feather * KH_2PO_4-0.001845 feather * agitation-0.032500 yeast extract * $CaCO_3$-3.08333 yeast extract * KH_2PO_4-0.021650yeast extract * agitation+1.42857 $CaCO_3$ * KH_2PO_4+0.005200 caco3 * agitation + 0.013167 KH_2PO_4 * agitation -0.395855 feather^2-6.04386 yeast extract^2-0.507662 $CaCO_3^2$-16.17172 $KH_2PO_4^2$-0.002195 agitation^2. Fisher's test and correlation coefficient (R^2) was calculated to identify the equation's fit quality. The relationship between the amino acid (Y) and the studied parameters' levels were illustrated by a 3D surface graphs according to **(Reddy et al 2008).**

Extraction and determination of total amino acids

Crude amino acids were collected by centrifuging culture at 10000 rpm under cooling for 15 min. Pellets were collected to determine cells dry weight and supernatant was used for amino acids identification analysis **(Lassoued et al 2015).** Determination of total amino acids was done as described by **(Anson, 1938).**

Chromatographic conditions for free amino acids analysis

The chromatographic conditions for analysis, using an Amino Acid Analyzer (Sykam, SW), equipped with a visible detector, was used for the individual free amino acid analysis. LC AKO6 Na - 24050313 column (4.6 mm 150 mm) was used for free amino acid determination. Immediately after injection onto the columns, an auto-sampler was used for the inline-derivatization by Ninhydrin (NIN) post-column derivatization. The NIN-derivatized amino acids were monitored at 570 nm and at 440 nm. The purchased standards of each individual amino acid (Sykam SW) were used for identification and quantification (external standard method). Individual free amino acid values were expressed as mg/l of the production medium.

Table 1. Media component levels studied in Plackett–Burman design

Variable Factors	Symbols	Coded levels	
		-1 (Low)	+1 (High)
Feather (g/l)	A	10.00	20.00
Glucose (g/l)	B	0.0000	10.00
yeast extract (g/l)	C	0.0000	2.00
Peptone (g/l)	D	0.0000	2.00
$CaCO_3$ (g/l)	E	3.00	10.00
KH_2PO_4 (g/l)	F	0.1000	1.20
Temperature (°C)	G	30.00	42.00
pH	H	6.00	8.00
inoculum size (%)	J	3.00	7.00
Agitation (rpm)	K	100.00	200.00
Incubation time (h)	L	24	72

Table 2. Plackett-Burman design model for media components optimization.

Run Order	(A) Feather (g/l)	(B) Glucose (g/l)	(C) yeast extract (g/l)	(D) Peptone (g/l)	(E) CaCO3 (g/l)	(F) KH2PO4 (g/l)	(G) Temp. (°C)	(H) pH	(J) inoculum size (%)	(K) Agitation (rpm)	(L) incubation time (h)
1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
2	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1
3	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1
4	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1
5	-1	+1	-1	+1	+1	-1	-1	+1	+1	-1	-1
6	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1
8	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
10	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1
11	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

Table 3. Media components levels in central composite (CCD) design

Variable	Symbol	Levels of the variables tested in CCD		
		-1 (Low)	0 (mean)	+1 (High)
Feather	(A)	10	15	20
Yeast extract	(B)	2	3	4
$CaCO_3$	(C)	3	6.5	10
KH_2PO_4	(D)	1.2	1.35	1.5
Agitation	(E)	100	200	200

Table 4. CCD design of the medium five components for optimization of amino acids production

Run Order	(A) Feather (g/l)	(B) yeast extract (g/l)	(C) CaCO3 (g/l)	(D) KH2PO4 (g/l)	(E) Agitation rpm
1	0	0	-1	0	0
2	+1	-1	+1	+1	-1
3	+1	+1	+1	-1	-1
4	0	0	0	0	-1
5	+1	+1	-1	-1	+1
6	+1	-1	-1	+1	+1
7	0	-1	0	+1	0
8	0	0	0	0	0
9	0	0	0	0	0
10	+1	+1	-1	+1	-1
11	0	0	0	0	0
12	-1	+1	-1	-1	-1
13	0	0	+1	0	0
14	+1	-1	+1	-1	+1
15	-1	+1	+1	+1	-1
16	0	+1	0	0	0
17	0	0	0	0	+1
18	0	0	0	0	0
19	-1	+1	+1	-1	+1
20	0	0	0	0	0
21	-1	0	0	0	0
22	0	0	0	0	0
23	+1	+1	+1	+1	+1
24	+1	-1	-1	-1	-1
25	+1	0	0	0	0
26	-1	+1	-1	+1	+1
27	-1	-1	-1	-1	+1
28	0	0	0	-1	0
29	0	-1	0	0	0
30	-1	-1	-1	+1	-1
31	-1	-1	+1	+1	+1

Amino acids production parameters

Productivity (P) = Amount of amino acids produced (gl^{-1}) / fermentation time (h) = $gl^{-1}h^{-1}$. Aqueous various concentrations of feathers disintegration (0, 5, 10, 15 & 20%) were autoclaved at 121˚C before used for cucumber plant treatments.

Plant material

Cucumber seeds (*Cucumis sativus L., cv. Beta Alpha*) were grown in plastic pots (150 mm diameter) filled with a mixture of sand and clay soil (1:1, v/v), one seed was sown per pot. The plants were watered daily, and fertilized once a week with 0.1% NPK (20:20:20). Plants were kept under normal greenhouse conditions.

Treatment and inoculation of *Podosphaera xanthii* on cucumber plants

Plant treatments after 28 days of planting (plants had the third fully expanded true leaves). In this stage plants were sprayed with different concentrations of amino acids (0, 5, 10, 15 & 20%), control plants were sprayed with distilled water. Ten replicates were used for each treatment. Conidia spores *of Podosphaera xanthii* were obtained from "eld-grown" plants, naturally infected cucumber leaves at field-grown cucumber plants in Qualubiya Governorate, Egypt, used as inoculum source. After 24h. from treatment, conidia were gently shaken to infect the upper surface of cucumber leaves. All plants were covered with polyethylene bags to maintain

relative humidity for 24h. An additional foliar spray was performed 13 days after inoculation.

Determination of disease index and sporulation

Disease index was assessed at the 11th and 20th days after inoculation (Twelve days after first treatment, and seven days after extra foliar application) visually using a 0-5 scale: where 0 = no disease symptoms, 1 = 1 – 10% of the host surface covered by mycelia, 2 = 11 – 15% of the surface covered by mycelia, 3 = 26 -50% of the surface covered by mycelia, 4 = 51 – 75% of the surface covered by mycelia, 5 = more than 75% of the surface covered by mycelia. Disease index of every plant was determined, according to the mathematical formula: Disease Index = {Σ a × b / (N * K)} × 100 % Where a is the number of leaves with a corresponding infection degree, b is the infection degree of leaves (scale differences from 0-5), N is the total number of leaves counted in a plant, and K is the maximal value of lesion intensity (= 5 on the chosen scale) **(Moradi et al 2017).** To calculate sporulation, ten leaf discs (9 mm in diameter) were collected from the second true leaf for all treatments. Conidia were lightly brushed in 5 ml ethanol (1%). After that, the number of conidia was counted using hemocytometer and expressed as conidia per cm^2 leaf tissue **(Reuveni et al 2000)**. The experiment was duplicated and the collected data were obtained from one typical experiment trial.

Biochemical changes in cucumber plants during foliar application of chicken feather hydrolysate

Extraction of plant crude enzymes

After 48 h of foliar application, one gram of the second true leaves were collected and grounded using a mortar and pestle under cooling in 2 ml extraction buffer composed of (5 ml of 1 M Tris-HCl pH 7.5, 0.2 ml of 0.5 M EDTA, 0.87 g sodium chloride, 1ml of DMSO up to 100 ml). Grounded samples were centrifuged at 10000 rpm for 10 min. at 4°C **(Conlon and Salter, 2007)**. The supernatant was transferred into new Eppendorf tubes to determine of peroxidase (POX), phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and superoxide dismutase (SOD). All enzymes activities were recorded as unit/ mg protein by using apparatus UNICO 2100UV Spectrophotometer. Lowry method for protein concentration calculations were used and bovine serum albumin was the standard protein **Lowry et al (1951)**.

Peroxidase activity assay

To determine peroxidase activity, 100 µl of the crude enzyme extract were added to a reaction mixture consists of 2.9 ml of sodium phosphate buffer (100 mM, pH 6.0) containing 0.25% (v/v) guaiacol (2-methoxy-phenol) and 100 mM H_2O_2 to100 µl of the crude enzyme extract. O.D. at 470 nm per min was used to measure absorbance of color change **(Hammerschmidt et al 1982).**

Phenylalanine ammonia-lyase activity assay

To measure the phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) activity, a reaction mixture of 1.9 ml of (0.05 M, pH 8.8) Tris-HCl buffer and 1 ml of (20 mM) L-phenylalanine was prepared, then 100 µl of the crude enzyme were added and incubated at 37˚C for 1 h. 0.2 ml of a freshly prepared HCl (6 M) was added to stop this reaction. Then, enzymatic activity was measured at 290 nm expressed as the L-phenylalanine conversion rate to trans-cinnamic acid **(Dickerson et al 1984)**.

Polyphenol oxidase activity assay

To test the activity of polyphenol oxidase (PPO), 100 µl of the crude enzyme extract were added to a 3.0 ml of a 0.01 M freshly prepared buffered catechol solution to 0.1 M phosphate buffer with pH 6.0). Absorbance of color change was measured at 495 nm **(Malik and Singh, 1980).**

Superoxide dismutase activity assay

To detect the superoxide dismutase activity, 200 µl of the crude enzyme extract were added to 3 ml of the prepared reaction mixture contained phosphate buffer (50 mM, pH 7.8), methionine (13 mM), nitro blue tetrazolium (75 µM), EDTA (100 mM) then, the addition of 2 µM riboflavin as the last ingredient in test tubes. All tubes were incubated under shaker of 100 rpm under light conditions for 10 minutes. After that, all tubes were covered with a black cloth to stop the reaction. Absorbance of the all reactions was read at 560 nm. **(Robert et al 1980).**

Statistical analysis

ANOVA with post hoc multiple comparisons using Tukey's HSD was analyzed to all data obtained from the all above experiments. For shake flask experiment trials, comparison of three replicates mean was calculated using SPSS 26.0 for windows at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

Statistical screening of significant media components by Plackett-Burman Design (PBD)

Six nutritional and five physical factors (feather, glucose, yeast extract, peptone, $CaCO_3$, KH_2PO_4, temp., pH, inoculum size, agitation rate and incubation time) were analyzed by PBD for their effects on amino acids production. Amino acids reached the highest production level at ratio of 2.1 g/l at run 7 which composed of (feather 20g/l, 0g/l of glucose, yeast extract and peptone, 10 g/l of CaCO3, 0.2 of KH_2PO_4, incubated at 42^{o}C at agitation rate of 200 rpm and inoculated with 2% of the standard inoculum. **Fig. 1** showed the main effects plot which is conjugated with ANOVA analysis to determine the level mean differences for all factors. The produced amino acids mean for each factor level was connected by a line. Factors with horizontal line parallel to the X axis are not main effects. However, factors with not horizontal line are considered as main effect factors. So, feather, yeast extract, $CaCO_3$, KH_2PO_4 and agitation rate are significant main effects for the amino acids production. ANOVA resulted that "Model *F*-value" equals to 26.4 and low probability value < 0.0500 (Prob. >F) indicated that the navigated model was significant. The variable parameters, revealing statistically remarkable significant results, were screened. Factors, revealing values of less than 0.05 (Prob. >F) which affected the response significantly were feather, yeast extract, $CaCO_3$, KH_2PO_4 and agitation rate as illustrated. Regression coefficient results showed that all process variables positively affect the amino acids production. However, effect of glucose, peptone, temp., pH, inoculum size and incubation time contribution was negligible. Therefore, these ingredients were avoided in the next step of optimization. Out of the significant media components, feather, yeast extract, $CaCO_3$, KH_2PO_4 and agitation rate, with confidence level 95%, were further optimized by CCD.

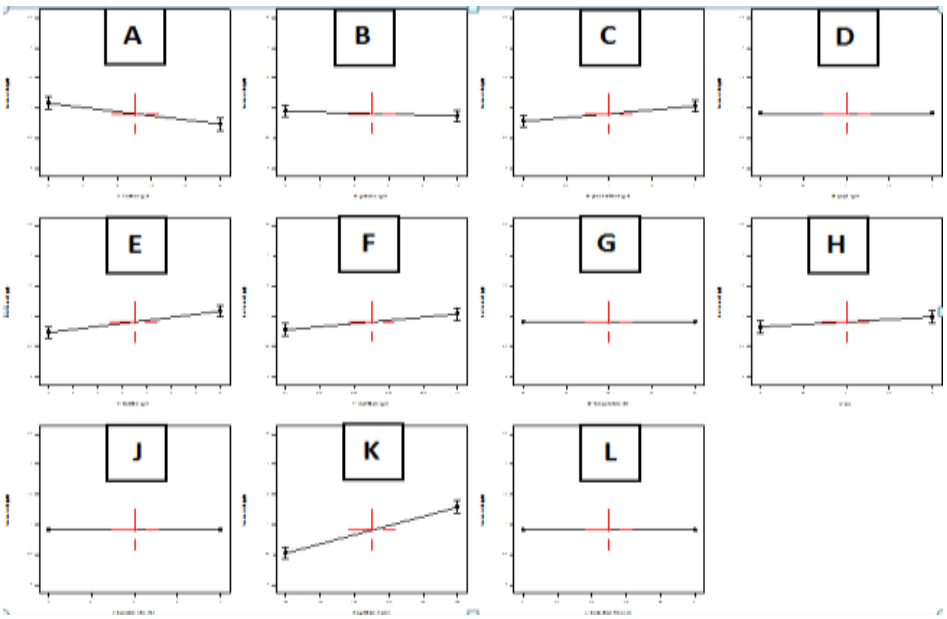


Fig 1. Main effects plot of PBD indicating that (feather: A, yeast extract: C, $CaCO_3$: E, KH_2PO_4: F and agitation speed: K) are the main significant media components affecting amino acid production by *Bacillus amyloliquifaciens 35s*

Central composite design (CCD) for optimization of medium components

The CCD statistical design tool using RSM was performed to study the singular and interactive effects of different process parameters on amino acids production by *B. amyloliquefaciens*. **Table 5** summarizes amino acids production of CCD experiment for each individual run along with the predicted response. Analysis of variance (ANOVA) was calculated to obtain the regression equation of the produced amino acids in which amino acids production (Y) =-142.21571+ 12.98718 feather+ 44.24693 yeast extract+5.56997 $CaCO_3$+78.35570 KH_2PO_4 + 0.786464 agitation +0.064500 feather * yeast extract-0.040571 feather * $CaCO_3$ -0.465000 feather * KH_2PO_4-0.001845 feather * agitation-0.032500 yeast extract * $CaCO_3$-3.08333 yeast extract * KH_2PO_4 - 0.021650 yeast extract * agitation + 1.42857 $CaCO_3$ * KH_2PO_4 + 0.005200 CaCO3 * agitation + 0.013167 KH_2PO_4 * agitation - 0.395855 feather^2-6.04386 yeast extract^2 - 0.507662 $CaCO_3^2$ - 16.17172 $KH_2PO_4^2$ - 0.002195 agitation^2. Model analysis of ANOVA was calculated as follows: the model *F*-value was 15.23. High model *F*-value with a very low probability value [(Prob. >F) less than 0.0001] indicated that the selected model was highly significant. The linear terms C, E, A^2, B^2, C^2, E^2 of the model were significant in which (Prob.> F less than 0.0500). The value of multiple correlation determination coefficients R^2 was 0.9018 which explains 90.18 % of the response variations. The model showed coefficient of variation (CV), standard deviation, mean and predicted residual sum of squares (PRESS) values of 9.69% 4.35, 44.91 respectively. For this model, the 'lack of fit' was found to be "not significant".

The software Design-Expert suggested solution runs based on the data generated with values lower and higher than the low and high levels of each factor for decreasing the experimental error. **Table 6** listed the observed optimum values of growth medium parameters and predicted response of the selected solution. The model regression equations were represented by using 3D surface graphs and 2D contour lines to represent the regression equation. These results showed that amino acids promotion conditions were different from those enhanced cell growth **(Venugoal and Saramma, 2006).**

In the present work, based on RSM study, various physical parameters and media components showed to have significant effects on amino acids production. Analysis of physical parameters indicated that production of amino acids by *B. amyloliquefaciens* is non-growth dependent. These results were previously observed by of Genckal and Tari (2006) Different variables yield were predicted from the respective response surface plots **(Fig. 2 A-C)**. Maximum level factors concentrations for maximum amino acids production was plotted using three dimensional (3D) response surface curves. **Fig 2** shows the relative effects of five different sets of factors, feather and yeast extract (A), feather and $CaCO_3$ (B), yeast extract and agitation (C), without change in the other factors' levels. Results clearly showed a strong degree of curvature of 3D surface, from where the optimum was determined. Model validation was done experimentally using CCD model for medium components. Model validation was verified as the actual and predicted values of 6.55 g/l, 6.60 g/l, respectively (as illustrated in **Table 6**).

Free amino acids content from hydrolyzed chicken feathers by amino acid analyzer

Release of free-amino acids in the modified medium inoculated with *B. amyloliquifaciens 35s* showed that there was an increase in the release of free amino acids (valine, glycine, leucine and thereonine) from the chicken-feathers as illustrated in **Fig. 3**. Amino acids were recovered through amino acid analyzer from the feather-hydrolysate in the following order: aspergine, therionine, serine, glutamine, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, and arginine.

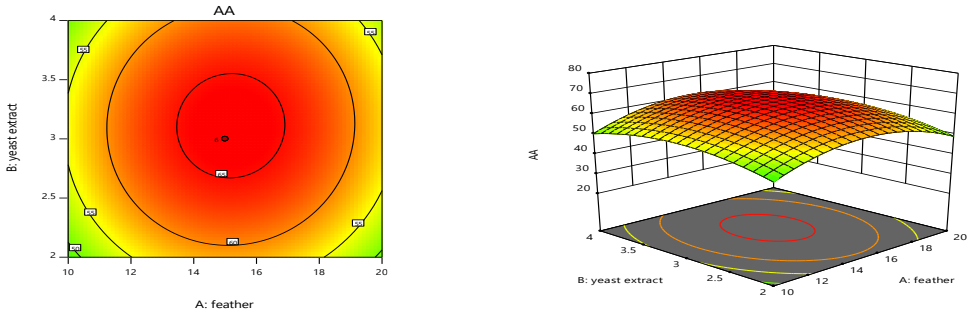
Table 5. Central composite design (CCD) of the three independent medium components with the observed and predicated values of produced amino acids

Run Order	(A) Feather (g/l)	(B) yeast extract (g/l)	(C) $CaCO_3$ (g/l)	(D) KH_2PO_4 (g/l)	(E) Agitation (rpm)	Actual Value (g/l)	Predicted Value (g/l)
1	15	3	0	1.35	150	3.54	3.63
2	20	2	10	1.5	100	3.58	3.66
3	20	4	10	1.2	100	3.07	2.78
4	15	3	6.5	1.35	50	3.16	3.03
5	20	4	3	1.2	200	4.33	4.69
6	20	2	3	1.5	200	6.50	6.60
7	15	3	6.5	1.65	150	2.57	2.51
8	15	3	6.5	1.35	150	6.55	6.60
9	15	3	6.5	1.35	150	4.22	4.56
10	20	4	3	1.5	100	6.55	6.60
11	15	3	6.5	1.35	150	6.02	5.28
12	10	4	3	1.2	100	4.69	4.45
13	15	3	13.5	1.35	150	3.82	3.84
14	20	2	10	1.2	200	4.04	4.42
15	10	4	10	1.5	100	5.43	4.80
16	15	5	6.5	1.35	150	3.35	3.19
17	15	3	6.5	1.35	250	6.55	6.60
18	15	3	6.5	1.35	150	3.58	3.44
19	10	4	10	1.2	200	6.55	6.60
20	15	3	6.5	1.35	150	6.55	6.60
21	5	3	6.5	1.35	150	6.55	6.45
22	15	3	6.5	1.35	150	3.69	3.80
23	20	4	10	1.5	200	3.87	4.05
24	20	2	3	1.2	100	3.16	3.54
25	25	3	6.5	1.35	150	3.75	3.88
26	10	4	3	1.5	200	3.44	3.49
27	10	2	3	1.2	200	3.54	3.63
28	15	3	6.5	1.05	150	3.58	3.66
29	15	1	6.5	1.35	150	3.07	2.78
30	10	2	3	1.5	100	3.16	3.03
31	10	2	10	1.5	200	4.33	4.69

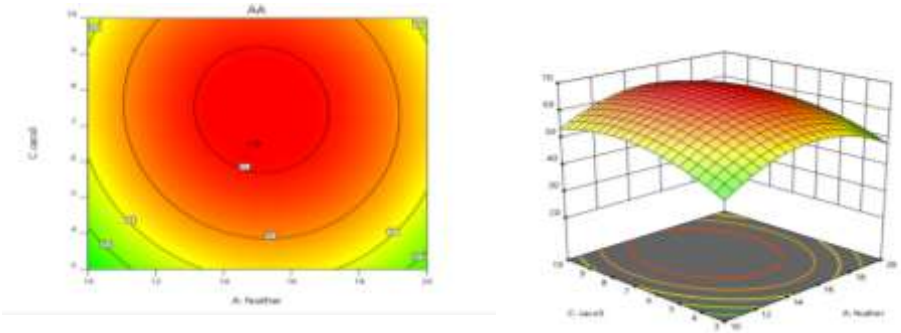
Table 6. Validation for the CCD model of medium components for optimized amino acids production by *B. amyloliquefaciens* 35s from chicken feather hydrolysate

Production parameters (and their levels)	Actual value (g/l)	Predicted value (g/l)	Biomass (g/l)	Productivity ($gl^{-1}h^{-1}$)	Productivity yield coefficient relative to biomass (%)
Feather (15g/l) Yeast extract (3g/l) $CaCO_3$ (6.5g/l) KH_2PO_4 (1.35g/l) Agitation (150 rpm)	6.55	6.60	14	0.27	46.78

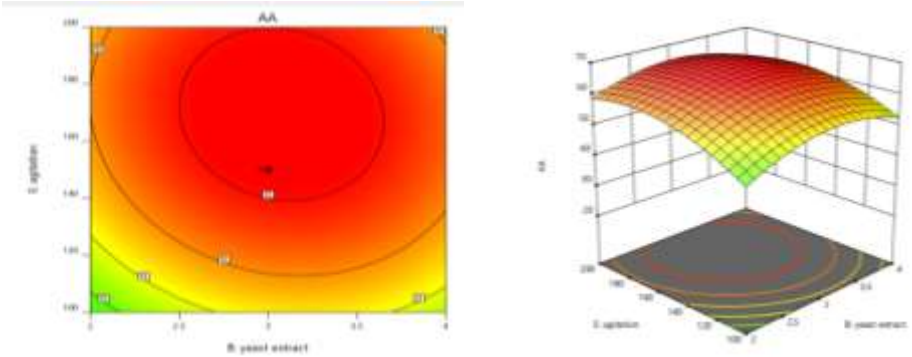
Productivity (P) = Amount of amino acids produced (gl^{-1}) / fermentation time (h) = $gl^{-1}h^{-1}$. Amino acids yield coefficient relative to biomass ($Y_{p/x}$) (%) = Amount of amino acids produced (gl^{-1}) / amount of biomass (gl^{-1}) *100.



(A) Contour and response surface plots showing the relative effects of feather and yeast extract on the production of amino acids.

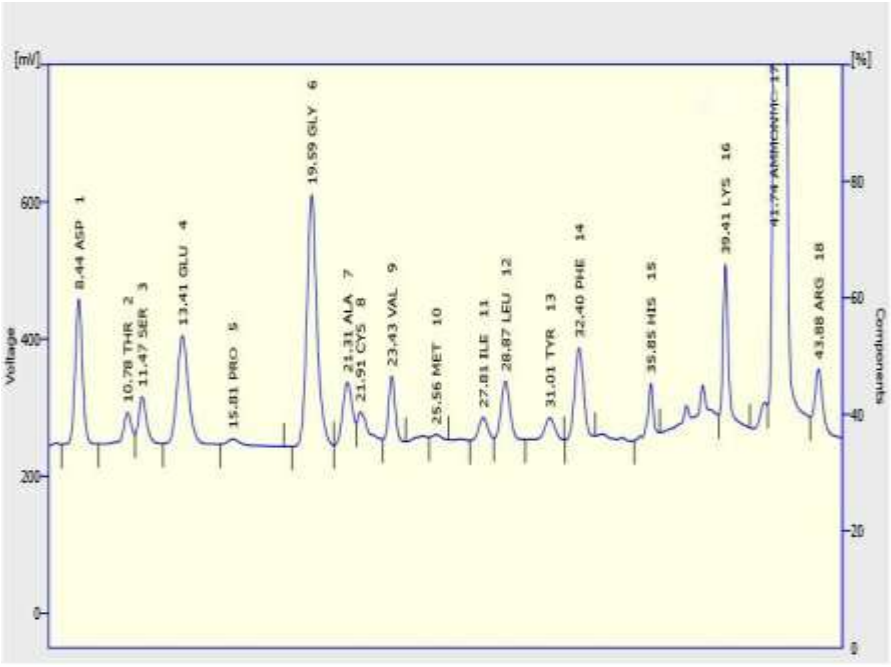


(B) Contour and response surface plots showing the relative effects of feather and $CaCO_3$ on the production of amino acids



(C) Contour and response surface plots illustrating the effects of yeast extract and agitation on the production of amino acids.

Fig. 2A-C. The two-dimensional contour plots and three-dimensional response surface plots showing the effect of nutritional factors and their mutual interactions on amino acids production by *Bacillus amyloliquifaciens 35s.*



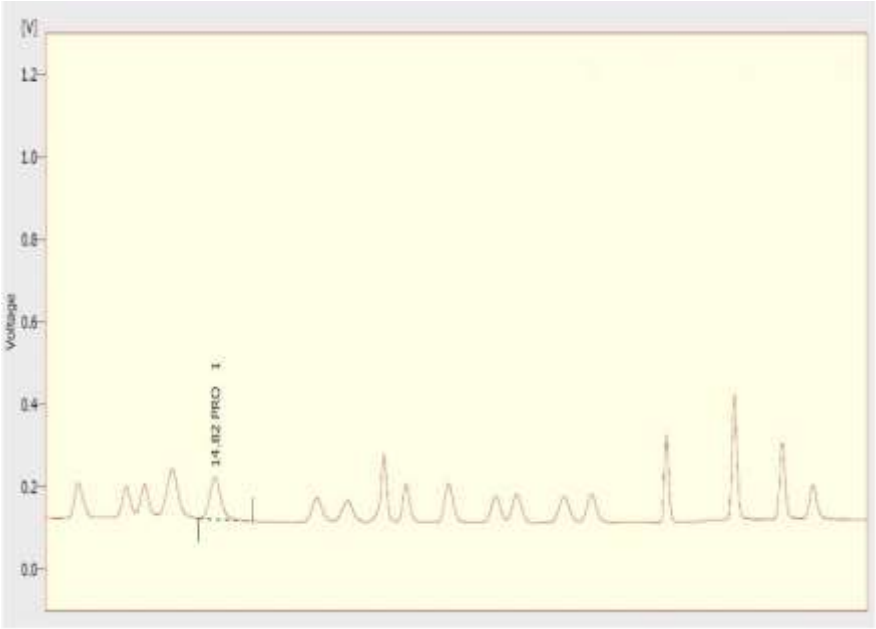


Fig. 3. Amino acids composition of chicken feather hydrolysate by automatic amino acid analyzer (Sykam SW) using LC AKO6 Na -24050313 column

Effect of chicken feather hydrolysate as foliar application on cucumber plants against *P. xanthii* the causing of powdery mildew disease

Foliar application using different concentrations (0, 5, 10, 15 and 20 %) of chicken feather hydrolysate were applied on cucumber plants post 24h. of infected plants with *P. xanthii.* Disease index was estimated on every plant. All concentrations of chicken feather hydrolysate showed significant effect on reducing disease index. The lowest concentration 5% was the lowest impact on disease. On the other hand, the higher concentration 20% showed the best effect on disease reduction compared with control plants. Extra foliar application was performed 13 days after inoculation showed a decrease in disease index for all concentrations compared with control plants **(Fig. 4, 5)**.

Also, all applied chicken feather hydrolysate concentrations led to a significant reduction in the number of conidia produced when compared to the control plants for both first and second foliar application treatments (**Fig. 6**). The mechanism by which this occurs is unknown possibly. However, amino acids contained in chicken feather hydrolysate might be effective in suppressing the disease. It could be concluded that chicken feathers have valuable source of natural amino acids which induced plant resistance against powdery mildew by increasing of antioxidant enzyme activity **(Moradi et al 2017).**

The results obtained reveal that foliar applications with higher amino acids concentrations from chicken feathers stimulated enzyme activities *i.e.,* peroxidase (POX), phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO) and superoxide dismutase (SOD).

Data illustrated in **Fig. 7** clearly indicate that there was direct relationship between POX activity and foliar concentration ranging between 10 – 20% Induction of POX may serve as a defense mechanism against powdery mildew disease as it contributes the exploitation of H_2O_2 in lignification of cells under both normal and stress conditions. Peroxidases (POX) catalyze many important biological processes of plant – defense mechanisms **(Gupta et al 2015)** as they play a significance role in strengthen of cell wall structures by catalyzing the suberin polymerization, and lignin biosynthesis. Plant peroxidases also facilitate the formation of diferulic acid linkages and production of hydroxyl radical **(Almagro et al 2009)**.

Data presented in **Figs. 8, 9 and 10** illustrated that all concentrations of foliar chicken feather hydrolysate (5, 10, 15, and 20%) stimulated significantly enzyme activities of PAL, PPO, and SOD

compared with untreated plants. The highest enzymatic activity was observed at concentration 20% of chicken feather hydrolysate. Phenylalanine ammonia-lyase (PAL) plays an important role as the key enzyme in phenol biosynthesis. It helps in plant resistance induction against different stress conditions through the regulation of phenolic compounds' biosynthesis **(Wen et al 2005)**. Phenylalanine ammonia-lyase converts phenylalanine to trans-cinnmaic acid which in turn modified by other enzymes resulting in phenolics including phytoalexins **(Singh and Prithiviraj, 1997).** Also, PAL activity is induced by several factors such as pathogen ingress, insect damage and abiotic stress.

Polyphenol oxidases (PPO) catalyses the o-hydrolxylation of monophenols to o-quinones, which rapidly polymerized to produce black or brown pigments (polyphenols). In healthy tissue, PPO are found in plastids and are responsible for tissue darkening symptom during lesion formation **(Vaughn and Duke, 1984)**. Their role is to limit secondary infection and the further spreading of pathogens by sealing off wounds or infected tissues. The oxidation of polyphenol by PPO formed a brown colored melanin which inhibits the growth of mildew disease. Plant PPOs oxidize polyphenols into antimicrobial quinones. Quinones are known to play an important role in lignifications of plant cell wall during pathogen's attack **(Tran et al 2012)**.

SOD is the first line of defense in plants by catalyzing superoxide anion radicles dismutation into hydrogen peroxide and molecular oxygen. Moreover, SOD induction reduces the cellular damage caused by oxidative stress **(Sunkar et al 2006)**.

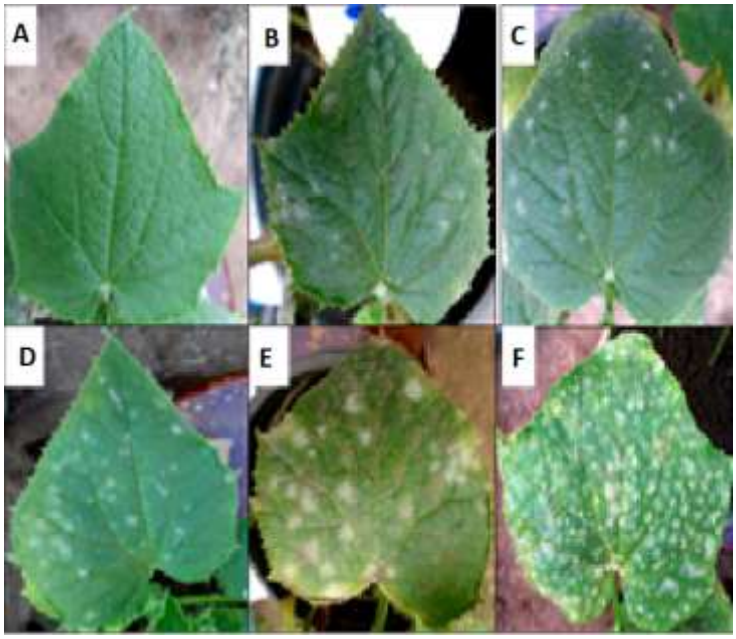


Fig. 4. Comparison of cucumber leaves treated with chicken feather hydrolysate against powdery mildew 20 days after inoculation with *P. xanthii*. (A): healthy leaf. (B): cucumber leave exposed to 20 % chicken feather hydrolysate. (C): cucumber leave with 15% chicken feather hydrolysate, (D): cucumber leave with 10 % chicken feather hydrolysate. (E): cucumber leave with 5% chicken feather hydrolysate (F): control. Photos were taken on the second true leaf of all concentrations.

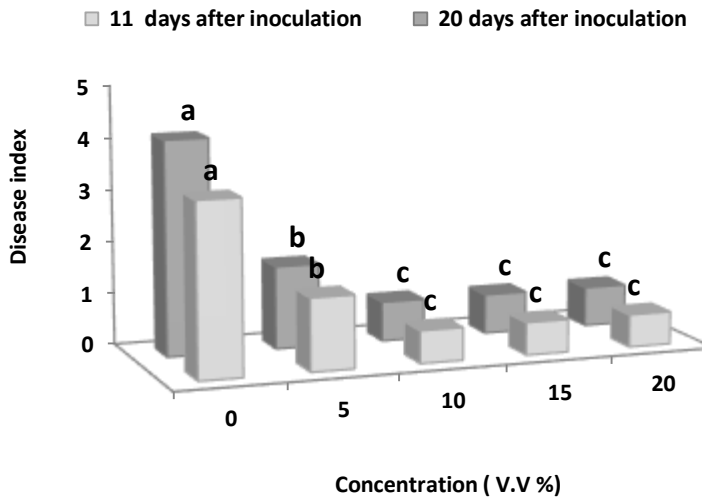


Fig. 5. Effect of foliar application of chicken feather hydrolysate concentrations on disease index of cucumber powdery mildew disease. Same letters show non-significant means.

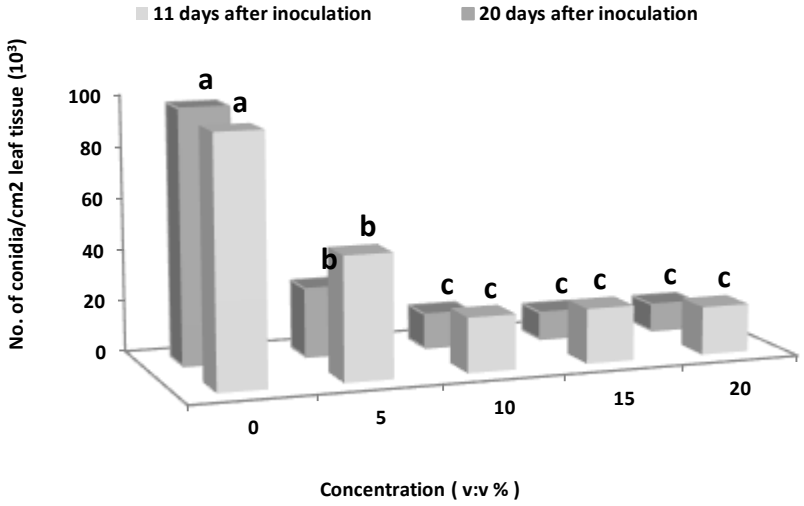


Fig. 6. Effect of foliar application of chicken feather hydrolysate concentrations on sporulation of *P. xanthii* on second true leaf of cucumber plant. same letters show non-significant means.

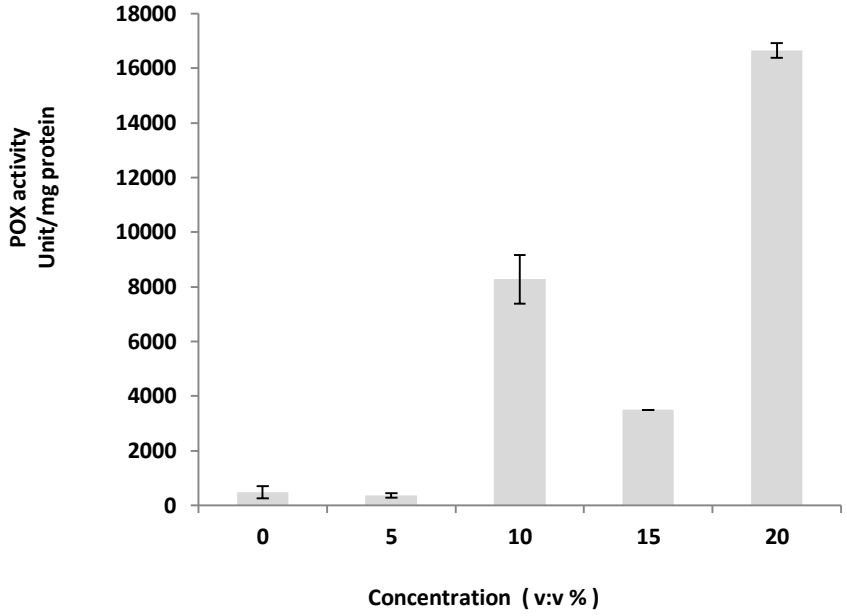


Fig. 7. Effect of foliar application of chicken feather hydrolysate concentrations on peroxidase activity of the second true leaf after 24 h. of inoculation.

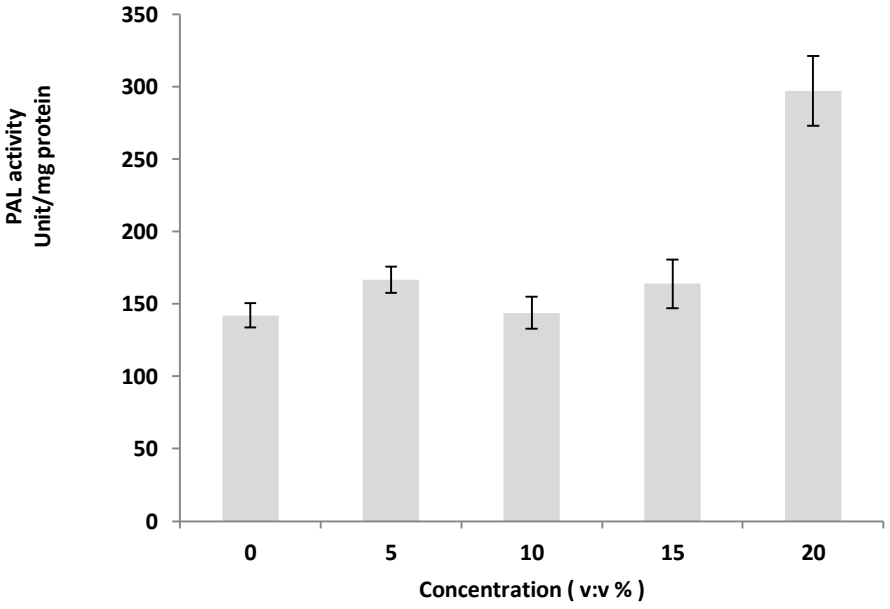


Fig. 8. Effect of foliar application of chicken feather hydrolysate concentrations on phenylalanine ammonia-lyase activity of the second true leaf after 24 h. of inoculation.

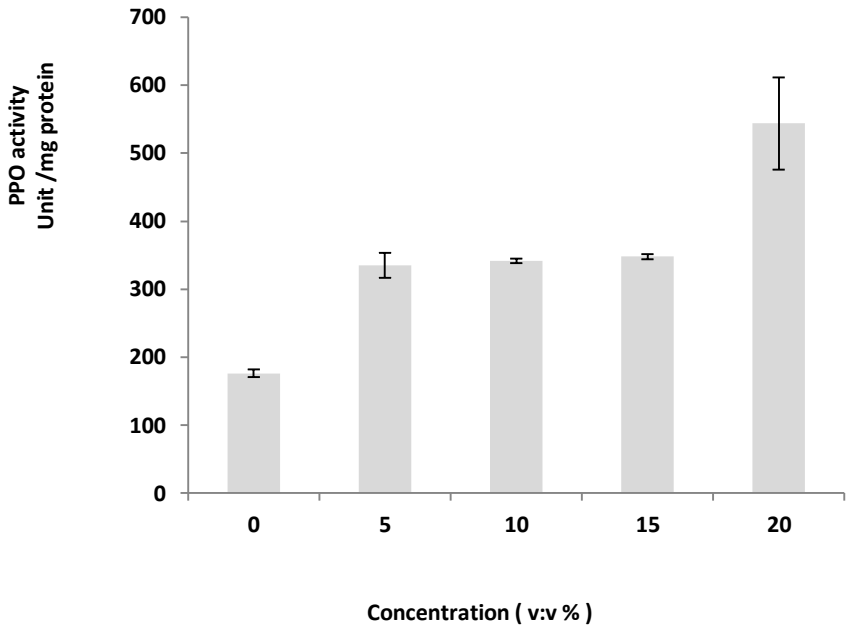


Fig. 9. Effect of foliar application of chicken feather hydrolysate concentrations on polyphenol oxidase activity of the second true leaf after 24 h. of inoculation.

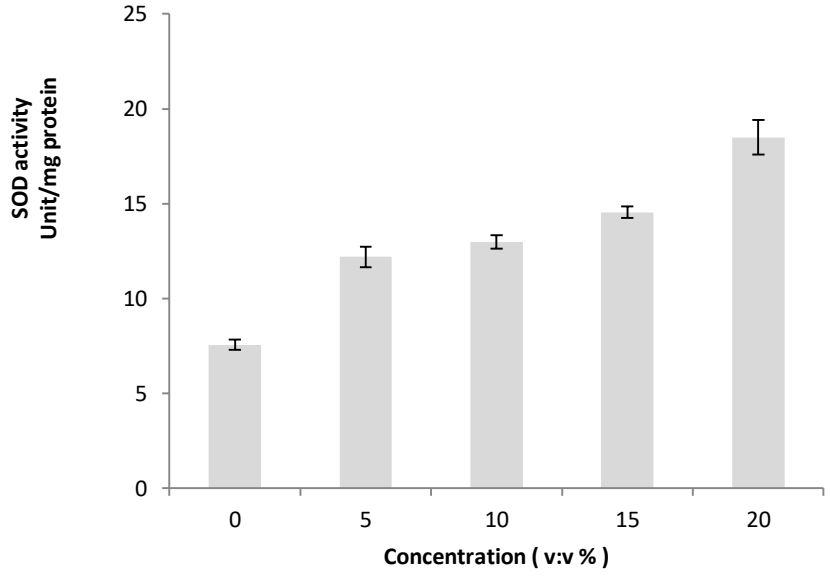


Fig. 10. Effect of foliar application of chicken feather hydrolysate concentrations on superoxide dismutase activity of the second true leaf after 24 h. of inoculation.

CONCLUSION

In this study, response surface methodology, as statistical approach, showed to be more adequate and efficient for the optimization amino acids production from chicken feather hydrolysate by *Bacillus amyloliquefaciens 35s.* Experimental analysis increased the amino acid production from 2.1 g/l in PBD by 3.1 times to reach 6.55 g/l. Amino acids analysis was performed using automated amino acid analyzer. Data showed the release of asparagine, threonine, serine, glutamine, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenyl alanine, histidine, lysine and arginine, respectively. Foliar application of cucumber leaves with feather chicken hydrolysate led to a significant increase in POX, PAL, PPO and SOD enzymatic activities as an indicator for a significant increase in the induced resistance of cucumber leaves against powdery mildew.

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تحسين إنتاج الأحماض الأمينية من ريش الدجاج بواسطة الباسيلس أميلوليكويفاشينز بإستخدام طريقةٌ RSM كطريقة جديدة لمقاومة مرض البياض الدقيقي

[59]

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الموجـــــــز

من خلال إستخدام تصميم Plackett Burman design (PBD) تم تحليل أحد عشر عنصرًا غذائيًا (ريش، جلوكوز، مستخلص خميرة، ببتون، كربونات كالسيوم، فوسفات بوتاسيوم ثنائية الهيدروجين، درجة الحرارة، الرقم الهيدروجيني، حجم اللقاح، معدل الرج وزمن التحضين) وذلك لتحسين إنتاج الأحماض الأمينية من ناتج تحلل ريش الدجاج ميكروبيا بواسطة *Bacillus amyloliquifaciens* 35s. أظهرت نتائج التحليل أن العوامل المؤثرة لإنتاج الأحماض الأمينية ميكروبيا ترجع إلى الريش، مستخلص الخميرة ، كربونات كالسيوم $CaCO_3$، فوسفات بوتاسيوم ثنائية الهيدروجين KH_2PO_4 ، ومعدل الرج agitation. أدى استخدام الطريقة الاحصائية Central composite design (CCD) إلى زيادة إنتاج الأحماض الأمينية من 2.1 جم/لتر في PBD بمقدار 3.1 مرة ليصل إلى 6.55 جم/لتر . أظهرت نتائج تحليل الأحماض الأمينية الناتجة من تحليل الريش بإستخدام جهاز amino acid analyzer انفراد كل من الاحماض الامينية التالية: الأسباراجين، الثريونين، سيرين، جلوتامين، برولين، جليسين، الانين، سيستين، فالين، ميثيونين، إيزوليوسين، ليسين، تيروزين، فينيل ألانين، هيستيدين، ليسين وأرجينين، على التوالي. تم تقييم التطبيق الورقي بتركيزات مختلفة (0 ، 5 ، 10 ، 15 ، 20%) من ناتج تحلل ريش الدجاج على الفطر المسبب لمرض البياض الدقيقي في نباتات الخيار المنزرعة تحت ظروف الصوبة عن طريق حساب درجة الاصابة والتجرثم. أظهر أقل تركيز لمنتج تحلل ريش الدجاج أقل تأثير على درجة الإصابة. على العكس من ذلك ، زادت مقاومة نبات الخيار بزيادة التركيزات المستخدمة وارتبطت بإنخفاض واضح في درجة الإصابة والتجرثم. معاملة الرش الورقي الإضافية أدت إلى خفض ملحوظ في تطور المرض لجميع التركيزات مقارنة مع النباتات الغير معاملة. وجد زيادة ملحوظة في نشاط الأنزيمات المؤكسدة المرتبطة بالمقاومة مثل بيروكسيداز (POX)، فينيل ألانين أمونيالياز (PAL)، بوليفينول أوكسيديز (PPO) وفوق أكسيد ديسموتاز (SOD) عند تطبيق التركيز المرتفع من راشح تحلل الريش.