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Kinetics of Enriched Chitinase as Extracellular Metabolite in *Beauveria bassiana*

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***Beauveria bassiana*, one of the most common entomopathogenic fungi, has been isolated, pre defined and characterized in-house from soil of tea cultivation area. Experiments have been performed to verify the presence of chitinase as intracellular metabolite and its release as extracellular product rendering the spores with biopesticide activity. Although there are many responsible enzymes for the pest killer action of *B. bassiana*, binding property of chitinase depending on presence as well as absence of serine supplemented in the media has been studied with respect to the production and kinetics. A programmed investigation conclusively indicates that the isolated spore (hyphae) of *B. bassiana* has been metabolically enriched with the enzyme chitinase in presence of an externally added amino acid serine with its inhibitory kinetics.**

Keywords: ***Beauveria bassiana*, chitinase, biopesticide activity, serine, inhibitory kinetics**

Introduction

With increase awareness for protecting environment from man-made pollution, use of chemical pesticides is being gradually restricted by the regulatory authorities of government agencies. In this situation, biopesticides are playing major roles in agricultural sectors. For this purpose, an attempt was made to isolate a naturally borne entomopathogenic fungi *Beauveria bassiana* from soil samples. *B. bassiana* secretes a group of enzymes viz., chitinase, lipase and protease which are the key biomolecules for biopesticide activity [1, 2]. The above group of enzymes either individually or collectively when come in contact with cuticle of pests, decompose the biochemical chitin present in cuticle. Such biochemical reaction renders *B. bassiana* its biopesticide property. Evidently more biopescticide activity will be observed due to enhanced release of chitinase.

In view of increased use of biopesticides in near future in agricultural sectors extensive investigation is now being carried out on searching newer sources of microorganisms having anti-pest activity and their reaction mechanism. Simultaneously, major emphasis is now being given to newer technologies for increasing the biopesticide activity of known microorganisms. With recent advances in biotechnology, genetically manipulated strains having high capacity of releasing chitinase can be a possible route in getting microorganisms enriched with biopesticide activity. However, such recombinant strains are subject to ethical, legal, risk and acceptability limitations.

As reported in the year 2004, *B. bassiana* Bb174 grown in wheat bran and silkworm chrysalis supplemented with peptone and other mineral nutrients produced 126 units/g chitinase with 2 days incubation at 28℃ [3]. In the consecutive year Weiguo *et al.*, cloned Bbchit1 (chitinase gene) of *B. bassiana* to improve the virulence activity against pest [4]. But, to explore the

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possibility of using amino acid precursor during the growth phase of *B. bassiana* for the purpose of increasing the content of the target chitin degrading enzyme is a first ever reported approach. This new route does not necessitate the application of recombinant technology in getting biopesticide property enriched microorganism. The microorganism selected in the present investigation is fungi *B. bassiana* with an intension to increase its chitinase content (one of the major enzyme responsible for chitin degradation) with the help of an externally added amino acid precursor serine during the growth phase. Fungal chitinases primarily come under the family 18 of the glycosyl hydrolase superfamily [5]. The basic structure of family 18 fungal chitinases comprises of five domains that shares the catalytic domain, N-terminal signal peptide region, chitin-binding domain, serine/threonine rich-region, and C-terminal extension region [6, 7]. On the other hand, serine/threonine-rich region acts as a linker between the catalytic domain and the chitin-binding domain and is required for maintenance of fungal chitinase stability [8]. It is felt that if serine is added as a precursor in the growth media, there would be possibility that the fungi *B. bassiana* will uptake it which increases the strength of catalytic domain of the serine rich region. The microorganism, *B. bassiana* will be metabolically modified with enriched chitinase content which makes the spores more biologically active towards degrading cuticles of pests. With this intension a programmed set of experiments have been carried out on the effect of externally added serine on the biopesticide activity of metabolically modified *B. bassiana* towards its anti pesticide activity.

Materials and Methods

Chemicals

Dextrose (Hi-media), peptone (Hi-media), yeast extract (Hi-media), agar for microbiology (Merck Limited, India), *B. bassiana* JUCHE22 (isolated by the present investigators from soil sample collected from Bogidhola TE, Golaghat district, Assam, India), deionized double distilled autoclaved water, Chitin flakes (C7170, Sigma-Aldrich Co., USA), hydrochloric acid (Merck Limited, India), N-acetyl glucosamine (NAG) (Sigma-Aldrich Co., USA), sodium phosphate buffer, potassium phosphate buffer, 2,4-dinitrosalicylic acid (DNS) (Merck Limited, India), phenol (Merck Limited, India), sodium sulphite (Merck Limited, India), sodium hydroxide (Merck Limited, India) and Rochelle's salt (Merck Limited, India) were used in the present experiment. 40% (w/v) solution of Rochelle's salt was prepared by adding 4 g of sodium potassium tartarate to 10 ml water.

Gene sequencing of isolated *B. bassiana*

The entire experiment was carried at Xcelris Private Limited, Ahmedabad (India). Gene sequencing of isolated and purified *B. bassiana* [8] was carried out using 28S rDNA sequencing. The D2 domain region of 28S rDNA amplicon of the reserved culture organism was carried using 570 bp (5'-AACCAACAGGGATTGCCCCAGTAA-3'>) forward primer and (5'-CAGCATCCT TGCGATGCGCGTACC-3'>) reverse primers. The sequence obtained was subject to NCBI BLAST search with the NCBI genebank database to find out the consensus sequence. The identified fungal isolate was studied for enhanced chitinase production with addition of commercial amino acid, serine as a precursor.

Media and Culture condition

The subcultured spores of *B. bassiana* JUCHE22 were grown in minimal media containing $MgSO_4$ (0.6 g/l), NaCl (0.5 g/l), $FeSO_4 \cdot 7H_2O$ (0.01 g/l), $ZnSO_4$ (0.01 g/l), KH_2PO_4 (1.0 g/l) with 1% colloidal chitin. One loop of spore culture was inoculated in 100 ml Erlenmeyer flasks. There were two duplicate flasks incubated at 25℃ for 5 days in total darkness to observe the screening method for chitinase production.

Colloidal chitin preparation

Colloidal chitin was prepared from partially purified chitin flakes of shrimps (Sigma). 40 gm of chitin flakes were ground and was dissolved in 400 ml of concentrated H_2SO_4 by stirring for 30 to 50 min. The chitin was precipitated as colloidal suspension by adding it slowly to 2 L of water at 10℃. The suspension was collected and washed by suspending it in about 5 liters of tap water by centrifugation. Washing was repeated until the pH of the suspension was about 7.0 [9].

Screening for chitinase

B. bassiana JUCHE22 was grown in minimal media containing 1% colloidal chitin and it was incubated at

25℃ for 5 days. After 5 days of incubation the culture was harvested, centrifuged at 1000 rpm for 15 min at 4℃ and the supernatant was collected. The plates of colloidal chitin agar (pH 7.0+/-0.2) were prepared containing K_2HPO_4 0.7 g/l; KH_2PO_4 0.3 g/l; $MgSO_4 \cdot 5H_2O$ 0.5 g/l; $FeSO_4 \cdot 7H_2O$ 0.01 g/l; $ZnSO_4$ 0.001 g/l; $MnCl_2$ 0.001 g/l, with moist colloidal chitin 10 g/l and 20 g/l agar was added as a solidifying agent [10]. A plain disc made from filter paper was dipped in the culture filtrate, and was placed in the agar, and incubated at 25℃ for 5 days keeping it in dark place. The development of clear zone around the disc was observed.

Experimental run

Sterile 100 ml minimal media containing chitin was transferred in a previously autoclaved 200 ml conical flask. one ml stock culture media was transferred to the conical flask in a laminar chamber. Metered quantity of serine solution was added to it as per the design of the experiment. The open mouth was plugged with sterile cotton ball. The system was kept in dark without shaking and the samples taken from this solution were analysed for its constituents.

Enzyme assay activity

The experiment was performed with DNS assay method [11]. 1% chitin was dissolved in 0.1 M sodium phosphate buffer at pH 8.0, DNS solution (1% DNS reagent, 0.5 g sodium sulphite, 1% NaOH, 2 g phenol in 1 L water), and 40% Rochelle's salt. 0.5 ml of the suspension and 0.5 ml of sodium phosphate buffer solution with dissolved 1% chitin was incubated at 37℃ for 45 min. After that 1.25 ml H_2O, 1.5 ml of DNS solution were added to it and heated for 12 min. The optical density of samples was measured at 540 nm with the help of a spectrophotometer (Specord 200 plus, Germany). The concentration of N-acetyl glucosamine was calculated with previously calibrated standard curve. One unit of chitinase activity defined as the amount of chitinase that catalyzed the release of 1 µmol of NAG per ml in 45 min [12].

Kinetic modelling

For the production of extracellular chitinase measured with respect to (N-acetylglucosamine) NAG can be described with the combination of chitin hydrolysis rate.

$$Chitin \xrightarrow[hydrolysis]{partial} N\text{-}chitobiose \xrightarrow[hydrolysis]{complete} N\text{-}acetylglucosamine$$

Since, the production of NAG is absolutely time dependent factor, it is necessary to determine the kinetics of the NAG production [13]. Initial rate of hydrolysis of the NAG production can be estimated with the help of Michaelis- Menten type saturation kinetics which can be described as follows:

$$V_0 = \frac{V_{\max}[S_0]}{K_M + [S_0]} \quad (1)$$

where, V_0 is the initial hydrolysis rate and K_M is the Michaelis constant and S_0 is the initial chitin concentration.

The time curves of NAG production were fitted to empirical equation (2),

$$NAG_m - NAG = NAG_m e^{-kt} \quad (2)$$

NAG_m is the maximum concentration of NAG produced and k is the first order rate constant [13].

$$K_i^{app} = \frac{K_i K_m}{K_m + [chitin]} \quad (3)$$

To determine the potential of the inhibition, it is essential to find out the value of inhibition constant K_i and K_i^{app}, the apparent inhibition constant.

Statistical analysis of experimental data

The experiment combinations with varying specified parameters (such as initial chitin concentrations, incubation time, serine concentrations) have been subjected to a set of 3 experimental runs to rigorous statistical analysis using Origin 2018b. In each case, the repeatability of 3 sets has been tested by the mean, and further interpreted by data analysis. The descriptive statistics and ANOVA so obtained by data processing have been used to generate the respective resulting figures.

Results and Discussion

Identification of the isolated *B. bassiana*

The phylogenetic tree obtained by 28S rDNA gene sequencing of the isolated strain is shown in Fig. 1A con-

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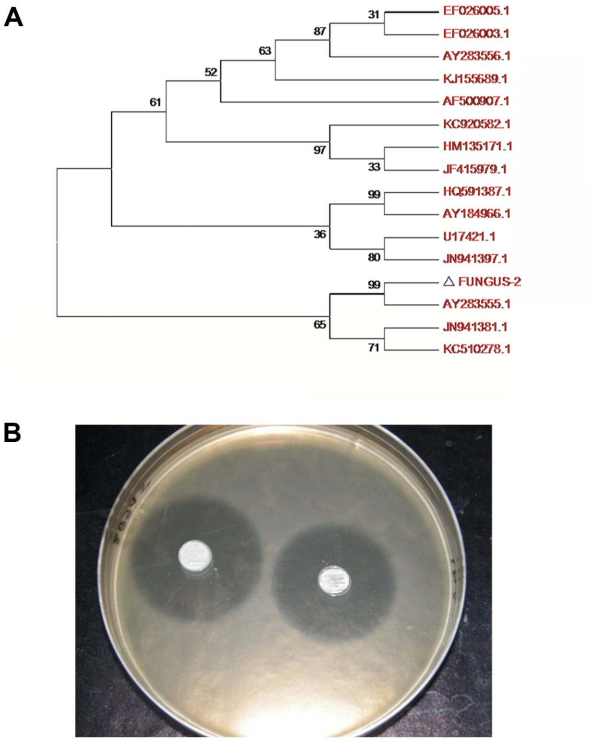

Fig. 1. (A) Phylogenetic tree of *Beauveria bassiana* strain, (B) Petri plate showing the chitin degradation.

sensus sequence of 600 bp is found to match with the maximum identity score. 28S rDNA sequence with the NCBI Nucleotide database with BLAST software revealed 99% sequence identity and the isolated strain has been listed as *B. bassiana JUCHE22*. The partial sequence obtained through gene sequencing experiment was submitted to the NCBI for inclusion in Nucleotide database which is accepted by NCBI and an accession number KT359346 is given to the strain.

In order to verify the presence of intracellular chitinase in the isolated strain and its release in the external microenvironment, screening experiment has been performed and the result is shown in Fig. 1(b). It is observed that chitinase has been released by the spores as one of the extracellular enzyme which visibly degrades chitin present in the media.

Enzyme activity in terms of NAG concentration of the isolated strain

Fig. 2 shows a plot of concentration of NAG as a function of initial chitin concentration. The final plot shows an appreciable confidence level ($R^2 = 0.98$) in Table 1. It is observed that concentration of NAG increases monotonically up to the initial concentration of chitin 36 g/l, and above this value, concentration of NAG drops steeply. Thus, this critical value (36 g/l) of initial concentration of chitin was maintained in further investigation.

In order to find out the effect of incubation time on the concentration of NAG released, experiments were performed at a fixed initial chitin concentration (36 g/l) without addition of serine and the result of dynamic response is shown in Fig. 3. Also, in Table 2, at 95% confidence level there is occurrence of p-value 0.98 for the given statistical data. The final plot shows an appreciable reliability level ($R^2 = 0.98$). It is observed that concentration of NAG rises rapidly with progress of time and attends the maximum value 82419.42 (µmol/l) on 13

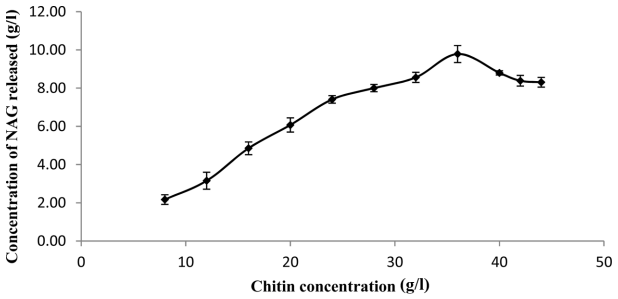

Fig. 2. Effect of chitin concentration on the release of (hydrolysed chitin) NAG.

Table 1. ANOVA Single factor.

Sum of squares	Degree of freedom	Mean of squares	p-value	F value	F-crit
1.445	2	0.722	0.89	0.116	3.315

Table 2. ANOVA Single factor.

Sum of squares	Degree of freedom	Mean of squares	p-value	F value	F-crit
0.277	2	0.138	0.98	0.010	3.554

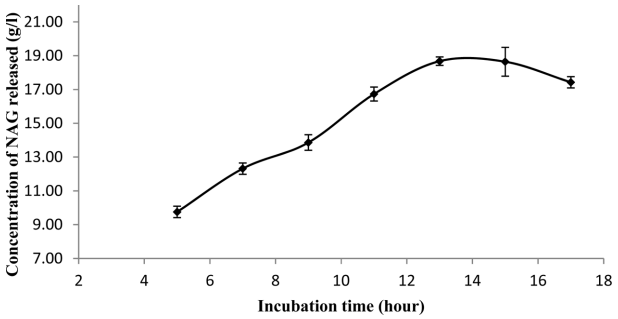

Fig. 3. Dynamic profile of NAG concentration.

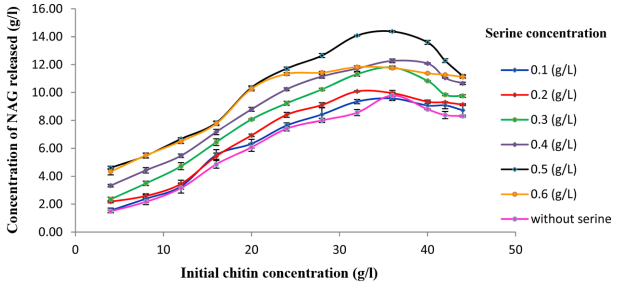

Fig. 4. Concentration of NAG (hydrolysed chitin) released against initial chitin concentration with serine concentration as parameter.

hours of incubation.

Studies on concentration of NAG in presence of precursor, serine

In order to find out the effect of the precursor serine on the concentration of NAG released, a set of programmed experiments were carried out with different initial serine concentrations ranging from 0.1 g/l to 0.6 g/l, and with different chitin concentrations ranging from 4 g/l to 44 g/l.

In order to get a quantitative comparison between these variables, concentration of NAG released has been plotted as a function of initial chitin concentration with concentration of serine as parameter, shown in Fig. 4. Table 3 shows that reliability (R^2) lie within the limit 0.99. Similarly, the p-value (0.99) shows a significant variance. For the purpose of comparison experimental data in absence of serine have been superimposed in the same figure. It is observed that release of NAG has significantly increased in presence of serine. A close inspection also reveals that with increase of serine concentration, concentration of NAG release increases for all the initial chitin concentration studied up to 0.5 g/l, beyond which release of NAG is reduced. The figure further reveals that at a particular serine concentration, concentration of NAG increases with increase in chitin concentration up to 36 g/l, but above this value, NAG release is reduced for all the initial serine concentrations studied. This observation evidently indicates that substrate inhibition of chitinase activity both in absence and presence of serine sets in at initial chitin concentration of 36 g/l.

Fig. 4 can also be used to arrive at a definite conclusion regarding the effect of serine on the release of NAG. It is apparent from the figure that release of NAG has been profoundly improved in presence of serine indicating the effectiveness of using serine as precursor. At initial incubation with optimized concentration of chitin (36 g/l) enzyme activity was increased from 42227.79 unit/l to 64619.14 unit/l on addition of 0.5 g/l serine. Moreover, on further increase of serine concentration there was an inhibition effect on the hydrolysed chitin with production of chitinase.

Time dependent variation of NAG from various concentration of chitin (24, 28, 32, 36, 40 g/l) in the presence of 0.5 g/l serine has been represented in Fig. 5. Repeatability of experimental data has been tested by taking 3 sets of experimental runs and in the present case, the final plot shows an acceptable confidence level (R^2) limit within 0.92 to 0.98, shown in Table 4. The concentration

Table 3. ANOVA Single factor.

Variance	Sum of squares	Degree of freedom	Mean of squares	p-value	F value	F-crit
0.1 (g/l)	0.099	2	0.049	0.99	0.005	3.284
0.2 (g/l)	0.230	2	0.115	0.98	0.013	3.284
0.3 (g/l)	0.230	2	0.115	0.98	0.013	3.284
0.4 (g/l)	0.067	2	0.033	0.99	0.003	3.284
0.5 (g/l)	0.054	2	0.027	0.99	0.002	3.284
0.6 (g/l)	0.038	2	0.019	0.99	0.002	3.284

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Table 4. ANOVA Single factor.

Variance	Sum of squares	Degree of freedom	Mean of squares	p-value	F-crit
36 (g/l)	0.061	2	0.030	0.92	3.315
32 (g/l)	0.013	2	0.006	0.98	3.315
28 (g/l)	0.152	2	0.076	0.92	3.315
40 (g/l)	0.054	2	0.027	0.97	3.315
24 (g/l)	0.057	2	0.028	0.95	3.315

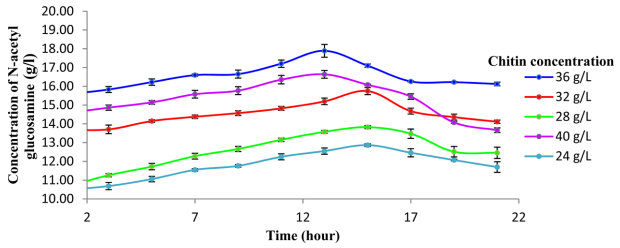

Fig. 5. Dynamic profile of NAG (chitin hydrolysed) in presence of serine (0.5 g/l).

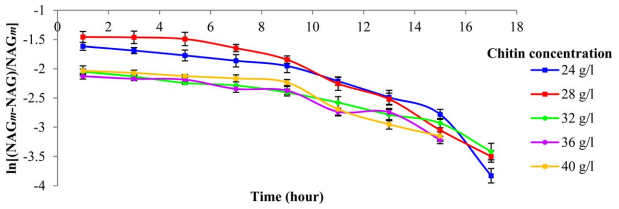

Fig. 6. Inactivation of chitin degradation with time.

of NAG gradually increased with increased chitin loading. However, further augmentation of chitin concentration (40 g/l) lead to reduced amount of NAG production. This phenomenon can be justified with the help of hydrodynamics. Higher concentration of NAG forms thick slurry which hinders the movement of the reaction system components which eventually obstructs the contacts between the enzyme and its substrate.

It has been observed from Fig. 4 that concentration of NAG released was increased till 20h and then decreased. The probable reason could be inferred that after a certain time period *B. bassiana JUCHE22* was not able to hydrolyze the provided concentration of chitin to NAG with the production of chitinase.

Saturation/Inhibition kinetics

In the present study, addition of serine as a precursor has shown an optimized increase in the production of chitinase with respect to hydrolysed NAG. Furthermore, in the present study, inhibition exerted by chitin (substrate) and values of various parameters (k, K_m, K_i) associated with it, are considered.

The value of K_m is 141.25 from the plot of $1/V_0$ against $1/S_0$, give the intercept $1/V_{max}$ and slope K_m/V_{max} which is quiet high. As the Michaelis constant represents the binding efficiency between the enzyme and its substrate, higher value of K_m represent that serine did not bind strongly with chitin and a slow increment of NAG was observed over a long period of time.

The time plot of NAG released were fitted to empirical Eq. (2), which has been considered as a first approximation in the treatment of data and presented in Fig. 6. The one-way ANOVA withdraw the significant values for the variance in the concentrations, where the probability of occurrence lies within 0.85 to 0.99 shown in Table 5.

Table 5. ANOVA Single factor.

Variance	Sum of squares	Degree of freedom	Mean of squares	p-value	F value	F-crit
24 (g/l)	0.114	2	0.057	0.89	0.113	3.402
28 (g/l)	0.023	2	0.115	0.98	0.017	3.402
32 (g/l)	0.055	2	0.027	0.85	0.159	3.402
36 (g/l)	0.002	2	0.001	0.99	0.007	3.466
40 (g/l)	0.007	2	0.003	0.97	0.022	3.466

Table 6. Variation of first order rate constant *k*/S and inhibition constant K_i ($m^{-1}s^{-1}$) with different chitin concentrations (g/l).

Chitin concentration (g/l)	*k*/s	K_i/m/s
24	0.21	0.00234
28	0.21	0.00240
32	0.23	0.00245
36	0.25	0.00251
40	0.25	0.00260

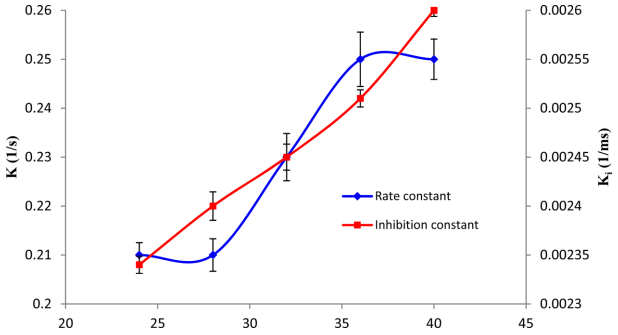

Fig. 7. First order rate constant and inhibition constant.

k in equation (2), is a function of chitin concentration, calculated from linear regression of the data obtained using various chitin concentrations with fixed intercept plotted against chitin concentration.

From Table 6, it was observed that with the augmentation of chitin concentration, value of *k* increased gradually. *k* represents the binding frequency between serine to its substrate and therefore it was assumed that with the increased chitin concentration, serine experienced higher amount of available chitin molecules and therefore, it can bind to its substrate more frequently. However, chitin concentration with 40 g/l, hinders the movement of the components of reaction system and hence hinders the frequency of the binding frequency of serine. For this reason, increment of chitin concentration from 36 g/l to 40 g/l did not further increase the binding frequency.

It is essential to find out whether, or not, the enzyme is inhibited by the consistent release of NAG. The value of K^i_{app} was calculated 0.002 from the slope of the plot rate constant *k*/s and chitin concentration (g/l). However, considering the value of K^i_{app} and K_m, the value of K_i was determined for each of the chitin concentration.

A higher inhibition constant (lower value of inhibition constant) associated with inhibition with higher potential. In the present study, Table 1 represents that with the increased chitin concentration the value of inhibition constant K_i gradually increases that means higher chitin concentration leads to lower inhibition. But although 40 g/l provides a lower inhibition, thick slurry formed during the reaction system play an inevitable role in inhibition. However, the difference among the values of inhibition constants was found very negligible for different chitin concentrations. Considering the absolute value of inhibition constants (0.002 till two decimal position of all inhibition constants) it was deduced that the value of inhibition constant is significantly low and therefore it can be concluded that serine is highly inhibited by the end product NAG as well as chitin. Variations in first order rate constant and inhibition constant with chitin concentrations is presented in Fig. 7.

Inference of statistical analysis

Data so obtained by statistical analysis used for plotting each figure (Fig. 2, 3, 4, 5, 6 and 7) are listed in Appendix I. The descriptive statistics evaluated the mean, standard error and confidence level (95%). The standard error for each point was calculated based on the mean of 3 experimental data for each figure. In Fig. 2, the overall standard error is 0.7, similarly, 1.3, 0.9, 0.2, 0.1 to 0.2, 0.008 (for rate constant) and 0.00044 (for inhibition constant) for Fig. 3, 4, 5, 6, 7 respectively. The selection of a confidence level for an interval determines the probability that the confidence interval produced will contain the true parameters. Also, the confidence level (95%) observed is 1.66, 3.34, 1.73 to 2.17, 0.41 to 0.62, 0.31 to 0.61 for Fig. 3, 4, 5, 6 respectively.

The ANOVA single factor estimated statistically significant differences between the means of three groups in this case of study. The source of variance was considered between the groups for sum of squares (SS) and mean square (MS). If the total sample means are close to each other then this will be small. There are k samples involved with one data value for each sample, so there are k-1 degrees of freedom (df). Moreover, we accepted the null hypothesis of all the experiments programmed because in all the cases, F value < F critical and p-value > 0.05.

Results obtained in the present investigation clearly indicate that the presence of externally added serine has

definitely metabolically modified the strain *B. bassiana* JUCHE22. The hyphae are capable of releasing greater quantity of NAG. This property has made them more active towards pests and thus the biopesticide activity has been considerably increased. Also we derived values of K_M (g/l), k/s, K_i/m/s obtained from experimental result. On the other hand the above values of inhibitory kinetics on addition of serine as a precursor to the optimized substrate concentration for the production of chitinase attained the first report that derived the interaction and rate of reaction. These calculated values proved the experimental enhancement of chitinase production with the release of NAG monomer in presence of chitin as well as serine concentrations.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Charnley AK. 2003. Fungal pathogens of insects: cuticle degrading enzymes and toxins. *Adv. Botanical Res.* **40**: 241-321.
2. Zacharuk RY. 1970. Fine structure of the fungus *Metarhizium anisopliae* infecting three species of larval Elateridae.III. Penetration of the host integument. *J. Invertebr. Pathol.* **15**: 372-396.
3. Zhang J, Cai J, Wu K, Jin S, Pan R, Fan M. 2004. Production and properties of chitinase from *Beauveria bassiana* Bb174 in solid state fermentation. *Ying Yong Sheng Tai Xue Bao.* **15**: 863-866.
4. Fang W, Leng B, Xiao Y, Jin K, Ma J, Fan Y, *et al.* 2005. Cloning of *Beauveria bassiana* chitinase gene *Bbchit1* and its application to improve Fungal strain virulence. *Appl. Environ. Microbiol.* **71**: 363-370.
5. Hayes CK, Klemsdal S, Lorito M, Di Pietro A, Peterbauer C, Nakas JP, *et al.* 1994. Isolation and sequence of an endochitinase-encoding gene from a cDNA library of *Trichoderma harzianum*. *Gene*. **138**: 143-148.
6. Seidl V, Huemer B, Seiboth B, Kubicek CP. 2005. A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. *FEBS J.* **272**: 5923-5939.
7. Takaya N, Yamazaki D, Horiuchi H, Ohta A, Takagi M. 1998. Intracellular chitinase gene from *Rhizopus oligosporus*: molecular cloning and characterization. *Microbiology* **144**: 2647-2654.
8. Arakane Y, Zhu Q, Matsumiya M, Muthukrishnan S, Kramer KJ. 2003. Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect. Biochem. Mol. Biol.* **33**: 631-648.
9. Mondal S, Datta S, Mukherjee A, Bhattacharya P. 2015. Studies on isolation, optimization and bioprocess engineering behaviour of entomopathogenic fungi, *Beauveria bassiana*. *Indian Chem. Eng.* **59**: 41-56.
10. Narayana KJP, Vijayalakshmi M. 2009. Chitinase production by *Streptomyces sp. ANU 627*. *Braz. J. Microbiol.* **40**: 725-733.
11. Murthy N, Bleakley B. 2012. Simplified method of preparing colloidal chitin used for screening of chitinase- producing microorganisms. *Int. J. Microbiol.* **10**: 2-7.
12. Miller GP. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*. **31**: 426-428.
13. Kuusk S, Bissaro B, Kuusk P, Forsberg Z, Eijsink VGH, Sørlie M, *et al.* 2018. Kinetics of H_2O_2-driven degradation of chitin by a bacterial lytic polysaccharide monooxygenase. *J. Biol. Chem.* **293**: 523-531.
14. Nagpure A, Gupta R. 2013. Purification and characterization of an extracellular Chitinase from antagonistic *Streptomyces violaceus-niger*. *J. Basic Microbiol.* **53**: 429-439.

APPENDIX I

Table of Descriptive statistics for Fig. 2.

Mean	Standard error	Median	Standard deviation	Sample variance	Confidence level (95%)
6.862	0.748	8	2.482	6.162	1.667

Table of Descriptive statistics for Fig. 3.

Mean	Standard error	Median	Standard deviation	Sample variance	Confidence level (95%)
15.625	1.367	16.731	3.618	13.095	3.346

Table of Descriptive statistics for Fig. 4.

Variation	Mean	Standard error	Median	Standard deviation	Sample variance	Confidence level (95%)
0.1 (g/l)	6.747	0.839	8	2.906	8.449	1.846
0.2 (g/l)	7.158	0.858	8.755	2.974	8.848	1.89
0.3 (g/l)	8.171	0.918	9.49	3.183	10.133	2.022
0.4 (g/l)	9.028	0.911	10	3.156	9.963	2.005
0.5 (g/l)	10.401	0.987	11.445	3.422	11.711	2.174
0.6 (g/l)	9.539	0.788	11.196	2.732	7.464	1.735

Table of Descriptive statistics for Fig. 5.

Variation	Mean	Standard error	Median	Standard deviation	Sample variance	Confidence level (95%)
36 (g/l)	16.5	0.192	16.26	0.637	0.405	0.427
32 (g/l)	14.5	0.185	14.376	0.616	0.38	0.413
28 (g/l)	12.503	0.301	12.513	0.99	0.996	0.67
40 (g/l)	15.286	0.282	15.45	0.937	0.878	0.629
24 (g/l)	11.764	0.233	11.76	0.774	0.6	0.52

Table of Descriptive statistics for Fig. 6.

Variation	Mean	Standard error	Median	Standard deviation	Sample variance	Confidence level (95%)
24 (g/l)	-2.158	0.235	-1.832	0.706	0.5	0.542
28 (g/l)	-2.14	0.267	-1.775	0.801	0.642	0.616
32 (g/l)	-2.507	0.138	-2.364	0.415	0.172	0.319
36 (g/l)	-2.488	0.129	-2.402	0.366	0.134	0.306
40 (g/l)	-2.431	0.144	-2.199	0.408	0.167	0.341

Table of Descriptive statistics for Fig. 7.

Variation	Mean	Standard error	Median	Standard deviation	Sample variance	Confidence level (95%)
Rate constant	0.23	0.008	0.23	0.02	0.0004	0.024
Inhibition constant	0.002	0.0004	0.002	0.0001	0.00001	0.0001

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