PRIMARY ARTICLE

Isolation Of Beauveria Bassiana, Classical Mutation And Solid Substrate Fermentation For Higher Biomass Production.

Chinnadurai Subramanian And Ganesh Punamalai

ABSTRACT

The study describes the isolation of *Beauveria bassiana* from soil sample collected from lower bhavani project areas of Tamil Nadu. Primary screening of *Beauveria bassiana* was examined by microscopic and macroscopic morphology and then confirmed by biochemical tests. Fast growing selected isolate are cultured for biomass production on submerged and solid state fermentation effected 20% packed cell mass in SMF and EOF cell mass of 22 grams by SSF. The selected isolate is irradiated to UV and conducted three rounds of mutation resulted. Thirty five mutants were screened for fast growth, increased enzyme activity and biomass production. The study has resulted mutant MUT-23 affected 35% PCV in SMF and 30 grams end of fermentation weight in SSF with lipase 45 u/g, protease 88 u/g and chitinase 34 u/g productivity.

KEYWORDS :

Beauveria bassiana, Spores, Solid state fermentation and Termites.

Chinnadurai Subramanian And Ganesh Punamalai
From
Department of microbiology, Annamalai University, Chidambaram, Tamil Nadu, India.

Te Article is published on September 2013 issue & available at www.scienceparks.in

DOI: 10.9780/23218045/152013/23

I. INTRODUCTION

Beauveria bassiana, an entomopathogenic fungi that grows naturally in soils throughout the world and acts as a parasite on various arthropod species, causing white muscardine disease. *Beauveria bassiana* kills the pest by infection as a result of the insect coming into contact with fungal spores. It is known as the white muscardine fungus because infected insect larvae eventually turn white or gray. It has an extensive host list that includes such important pests as whiteflies, aphids, grasshoppers, termites, Colorado potato beetle, Mexican bean beetle, Japanese beetle, boll weevil, cereal leaf beetle, bark beetles, lygus bugs, chinch bug, fire ants, European corn borer, codling moth and Douglas fir tussock moth. The species was named after the Italian entomologist Agostino Bassi, who discovered it in 1835 as the cause of the muscardine disease of domesticated silkworms. It was formerly also known as *Tritirachium shiotae*. *Beauveria bassiana* is the anamorph (asexually reproducing form) of Cordyceps bassiana. The latter teleomorph (the sexually reproducing form) has been collected only in eastern Asia.

Among them, biological control is one that received great interest among researchers (Milner & Staples 1996, Grace 1997). *Beauveria bassiana* (Balsamo) Vuillemin is a naturally occurring entomopathogenic fungus with a very wide host range (Tanada and Kaya, 1993). Comparisons among different *B. bassiana* isolates and with other entomopathogenic fungi (Lai *et al.*, 1982; Delate *et al.*, 1995; Jones *et al.*, 1996; Neves and Alves, 2000) revealed differences in isolate virulence against termites. Entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae* have been reported to be highly active under laboratory conditions against *Triatoma infestans* and other triatomine species (Romana and Fargues, 1987; Luz *et al.*, 1998 a, b; Luz and Fargues, 1999; Fargues and Luz 2000; Lecuona *et al.*, 2001) and to reproduce on their cadavers (Luz and Fargues 1998; Fargues

and Luz, 1998).

Beauveria bassiana has a variety of characteristics that make it unique to other pathogens. It occurs naturally in soil throughout the world. It reproduces sexually and asexually. The conidia adhere to the insect cuticle, germinate and penetrate in the insect body, where they replicate as yeast-like cells (blastospore) and destroy the internal structures, causing morbidity within 36 - 72 hours. After death of the insect, the blastospore transform into mycelia, which emerge through the cuticle and form spores. These spores are viable and turn into yellow color. *Beauveria bassiana* also has no preference as to its host stage in life cycle. It will attack larvae and adults. A very unique characteristic is that it affects its host upon contact, unlike many other pathogens that need to be consumed to cause infection. Upon contact the pathogen kills the host from the inside out.

It produces spores, known as conidia (asexual form), that directly infect through the outside of the insect's skin, then proceeds to germinate. The conidia of *B. bassiana* adhere to the insect cuticle intersegment membrane by means of hydrophobic interaction between the spore wall and epicuticle lipids. The conidia germinate and the germ tube penetrates the cuticle, using a specific series of enzymes like lipase, protease and chitinase, which in turn degrade the lipids, protein and chitin in the insect cuticle. In the insect body, the fungus multiplies in the haemocoel as a blastospore, or yeast - like cell and enzymes begin to destroy the internal structures of the host insect causing morbidity within 36 - 72 hours. It also produces Beauvericin, a toxin that weakens the host's immune system. At this point, the fungus was replicating and feeding on the host's internal organs such as gastrointestinal, malpighian tubules, fat body and blood - like fluids. The host dies within three to seven days after contact. Reduced feeding and immobility are rapidly evident, and the insect dies within between 4 to 10 days post - infection. The time to death will depend on the insect species, age and conidial dose.

The conidia of *Beauveria bassiana* in host epidermal or porosity, digestive tract, in appropriate conditions began to germinate, born of germ tube. Simultaneously, it produce lipase, protease, chitinase dissolved the insect cuticle, pipe intrusion body from bud, growth in insect reproduction, consumption of the host nutrients, the formation of a large number of hypha and spore, full of body whole body. At the same time, produce a variety of toxins, Beauvericin *Beauveria brongniartii* hormone (Tenellin) and oospores hormone (Oosporein). White muscardine prime for the cyclic peptide N - containing methyl amino acid. White muscardine prime for the needle colorless crystals, with lead acetate in aqueous solution can produce flocculent white precipitate, three positive reactions of indene ketone derivatives. It also produce white muscardine hormone (macrocyclic lipid toxin) and calcium oxalate crystals, these substances can cause the insects to poisoning, make the body found function changes, disrupted the new supersedes the old and leads to death.

In the present study, we isolated the wild *Beauveria bassiana* from infected termites and optimize the biomass production in solid substrate production by using various cost effective substrates and attempt on conventional mutation methods under UV radiations exposure to various time course and screen for the fast growing and higher cell mass producing mutants under lab scale submerged fermentation and Lab Koji based Solid substrate fermentation studies. The lipase, protease and chitinase activities were established to evaluate the conidia germination and the germ tube penetrates the cuticle, using a specific series of enzymes like lipase, protease and chitinase to degrade the lipids, protein and chitin in the insect cuticle.

2. MATERIALS AND METHODS

Termites are important urban pests which can cause a tremendous amount of damage to homes and structures. Prevention of termite damage has been a challenge because of their large populations and cryptic behavior. Various methods in termite control were explored in the past including physical, cultural, chemical, and biological methods. The biological method includes the usage of *Beauveria bassiana* to control the termites. Fifteen different soil samples are collected in sterile polythene bags from various farms of lower

bhavanai cultivation areas of Tamil Nadu and transferred to R&D division. The samples are dispersed into sterile water and shaker for 10 - 20 minutes.

2.1. Primary screening

The primary screening media contains 5g/L yeast extract (Hi Media), 10g/L Maltose (Hi Media), 5g/L Glucose (Hi Media), 6g/L Potato infusion, 2g/L Soya peptone (Hi Media), 2.5g/L Dextrose (Hi Media), Agar (Hi Media) and 30g/L agar (Hi Media). All the ingredients are weighed and dissolved in reverse osmosis water and autoclaved at 121°C at 15psi pressure for 30 min. The homogenized samples are inoculated and incubated at 27°C for 5 days and the examined for morphology. It was observed that some of the colonies are white color powdery fungi. Further, templates are created and incubated at 27°C for 5 days. A loop full of culture of both at vegetative phase and sporulation phase are prepared with thin smear and stained with Lactophenol cotton blue (LPCB), viewed under microscope under oil immersion magnification (100x).

Figure - 1

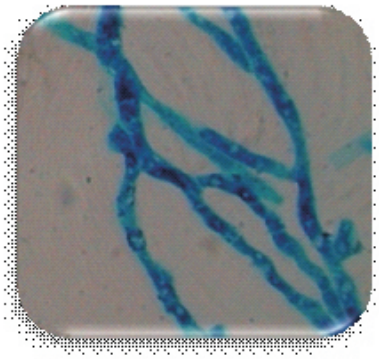


Figure - 2

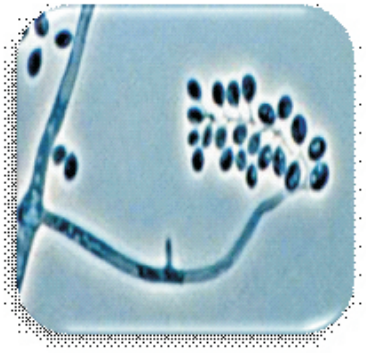


Figure - 3

Figure - 1 represent the colonies produce many dry, powdery conidia in distinctive white spore balls. Figure - 2 represents the mycelia or vegetative stage of growth; it is usually filamentous and has definite cell walls. The growth rate is moderately rapid. The colonies attain 3 cm diameter and following incubation time of seven days at 27 degrees Celsius on potato glucose agar. Seven colonies are isolated and further the templates are created and tested for fast growing colonies for enhanced virulence, pathogenicity and host range on termites. Figure - 3 represents the spore ball was composed of a cluster of conidiogenous cells. The conidiogenous cells of B. bassiana are short and ovoid and terminate in a narrow apical extension called a rachis. The conidia are single - celled, haploid, and hydrophobic

Table – 1: List of *Beauveria bassiana* Isolates

Isolate No	Initial Colonization log (hrs)	Size of colony 48 (hrs) cm	Size of colony 72 (hrs) cm
IS-1	10	2.8	3.4
IS-2	14	2.4	2.8
IS-3	12	2.2	2.6
IS-4	16	2.3	2.7
IS-5	18	1.8	2.5
IS-6	16	2.5	2.9
IS-7	18	1.8	2.6

IS-1 was selected as fast growing isolate and grown in submerged seed media of following composition Glucose (Hi Media), 10g/L, Soluble starch 15 g/L (Sdfin), 5 g/L Soya flour defatted toasted (commercial) 5g/L yeast extract (Hi Media), 10g/L Maltose (Hi Media) 700 ml in 2000 L flask and incubated at 248 RPM at 27°C for 4 days. The end of fermentation pH was 4.5 and packed cell volume is 20% at 10000 RPM for 10

minutes. The solid state media was prepared with cost effective substrate baggase treated with 2% glucose and 2% seed media feed. The solid substrates 10 g were weighed and 3 ml of water was added. It was mixed thoroughly and packed 10 g in Koji plates. The Koji plates were autoclaved at 121°C at 15psi pressure for 30 min. It was inoculated with 10 ml of IS 1 to 7 respectively and incubated for 6 days under static at room temperature. The colonization on plates was monitored for every 6 hrs. The plates are harvested at 6 days effected 23 grams end of fermentation weight with 53% moisture. The end of fermentation koji was harvested and from which 3 grams was extracted in sterilize demineralised water and tested for enzyme activity for protease using Casein as a substrate (Sigma-Aldrich Method), Lipase assay using tributyrin substrate (Sigma-Aldrich Method) and Chitinase Assay using 4 - Nitrophenyl N, N – diacetyl – β - D- chitobioside substrate (Sigma-Aldrich Method) and the results are presented in Table - 2. The conidia of *Beauveria bassiana* in host epidermal or porosity, digestive tract, in appropriate conditions began to germinate, born of germ tube. Simultaneously, it produce lipase, protease, chitinase dissolved the insect cuticle, pipe intrusion body from bud, growth in insect reproduction, consumption of the host nutrients, the formation of a large number of hypha and spore, full of body whole body. Hence, the enzyme production is important for the better activity against termites.

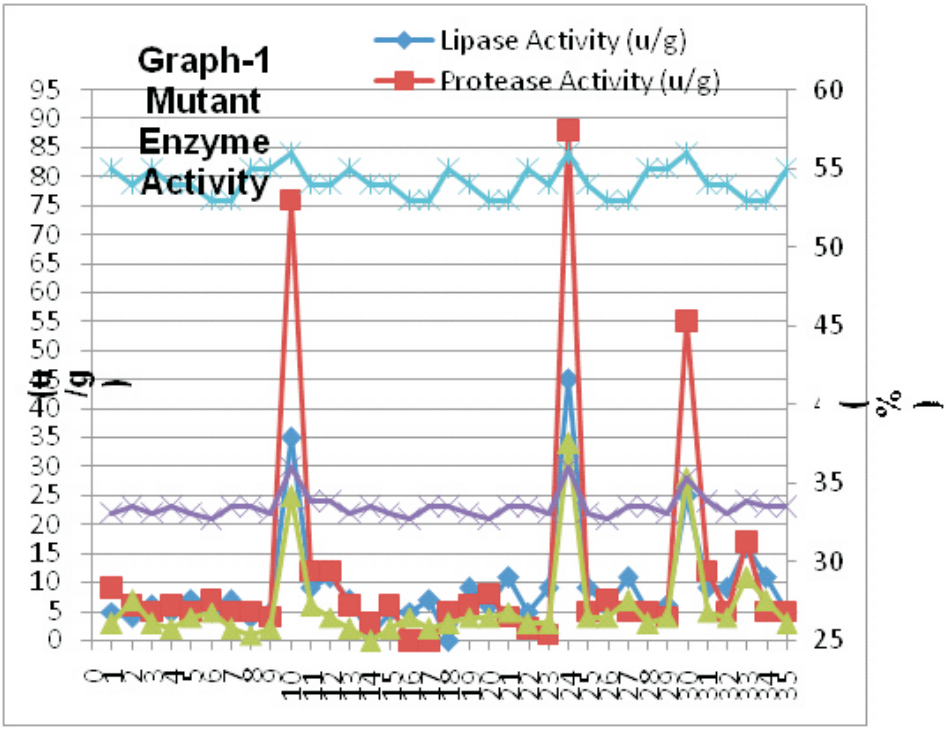
In order to increase the cell mass production and over all enzyme activity the IS-1 was subjected for random wise classical mutation through UV mutation for spores. The spores are suspended in Phosphate buffer pH 6.5 and counts are tested in hemocytometer and selected for mutation. The spore suspension serially diluted to 10-2 prepared in 9 eppendorf tubes, spun, supernatant decanted and the pellet is suspended in phosphate buffer and exposed to UV radiations under agitations for various log hrs that includes 10, 20, 30, 40, 60, 120, 140 to establish the maximum kill rate. It was found that 120 minutes UV exposure affected more than 99 % kill rate and hence the procedure was standardized with 120 minutes UV exposure. The plates are incubated at dark conditions for 5 days. Three rounds of mutation affected more than 99% kill rate and 45 mutants are selected for the evaluation to check enzyme activity and increased biomass production and results are provided at Table – 4.

Table – 2: Enzyme activity of *Beauveria bassiana* Mutants

Isolate No	Lipase (u/ml)	Protease (u/ml)	Chitinase (u/ml)	EOF Volume (g)	EOF Moisture (%)
Wild	5	9	3	22	55
MUT-1	4	6	7	23	54
MUT-2	6	5	3	22	55
MUT-3	5	6	2	23	54
MUT-4	7	5	4	22	54
MUT-5	5	7	5	21	53
MUT-6	7	5	2	23	53
MUT-7	4	5	1	23	55
MUT-8	5	4	2	22	55
MUT-9	35	76	25	30	56
MUT-10	9	12	6	24	54
MUT-11	11	12	4	24	54
MUT-12	7	6	2	22	55
MUT-13	1	3	0	23	54
MUT-14	5	6	2	22	54
MUT-15	5	0	4	21	53
MUT-16	7	0	2	23	53
MUT-17	0	5	3	23	55
MUT-18	9	6	4	22	54
MUT-19	6	8	4	21	53
MUT-20	11	4	5	23	53
MUT-21	5	2	3	23	55
MUT-22	9	1	3	22	54
MUT-23	45	88	34	30	56
MUT-24	9	5	4	22	54
MUT-25	6	7	4	21	53
MUT-26	11	5	7	23	53
MUT-27	5	5	3	23	55
MUT-28	6	4	4	22	55
MUT-29	25	55	28	28	56
MUT-30	9	12	5	24	54
MUT-31	9	5	4	22	54
MUT-32	16	17	11	24	53
MUT-33	11	5	7	23	53
MUT-34	5	5	3	23	55
MUT-35	9	11	6	22	55

Figure – 4: MRL/MUT-23

The following three mutants are shortlisted MUT-9, MUT-23 and MUT-29 on which MUT-23 is selected as the best and higher potential. MUT-23 was selected as potential mutant most suitable for the solid state fermentations methods and the Figure - 4 represent the cell mass production.



3. CONCLUSION

UV irradiation of Beauveria bassiana spores under agitated exposure affected more than 99% kill rate on which a mutant labeled MUT-23 has affected Lipase 45 u/g, Protease 88 u/g and Chitinase 30 u/g with increased biomass production of 30 grams with the initial addition of 10 gram baggase and moisture 3 gram and inoculum 10 ml of 20% pcv. The colonization in solid substrate was found to be most suitable in biomass generation by using cost effective baggase as substrate and confirms that it can be mass - produced on locally available baggase and other solid substrates.

REFERENCES

1)Changlu Wang and Janine E. Powell. Isolation and evaluation of Beauveria bassiana for control of Coptotermes formosanus and Reticulitermes flavipes (Isoptera: Rhinotermitidae). Sociobiology, 41(1):
2002.

2)Robledo-Monterrubio, R Alatorre-Rosas, G Viniegra-González, O Loera Selection of improved Beauveria bassiana (Bals.) Vuill. strains based on 2-deoxy-D-glucose resistance and physiological analysis. Journal of Invertebrate Pathology, 101(3):222-7 06/2009.

3)Meyling NV, Eilenberg J.Isolation and characterization of Beauveria bassiana isolates from phylloplanes of hedgerow vegetation. Mycol Res., 2006 Feb;110(Pt 2):188-95. Epub 2005 Dec 27.

4)Dwayne D. Hegedus, George G. Khachatourians Isolation and characterization of conditional lethal mutants of Beauveria bassiana Canadian Journal of Microbiology Volume 40, Number 9, September 1994

5)Thomas, K. C., G. G. Khachatourians, W. M. Ingledew Production and properties of Beauveria bassiana conidia cultivated in submerged culture Canadian Journal of Microbiology Volume 33, Number 1, January 1987