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IN VITRO STUDIES ON SUITABILITY OF VARIOUS NATURAL AND SYNTHETIC SUBSTRATES FOR GROWTH, CELL BIOMASS AND CRYSTAL PROTEIN PRODUCTION OF *BACILLUS THURINGIENSIS* ISOLATE PKKVK 9

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ABSTRACT

The gram positive endospore forming *Bacillus thuringiensis* bacterium producing pesticidal crystal proteins known for encountering the insect pests is considered to be environmentally safe. Several methods used to prawl more number of infective colonies, they may vary on production techniques using solid substrates by the process Media Optimization. The low cost and ease of preparation of agro-industry based wastes and organic substrates having carbon and nitrogen sources in addition to organic basal salts supporting *Bacillus thuringiensis* growth profusely. Utilizing certain organic raw materials and synthetic substrates optimally to increase the cell biomass and crystal protein production and iniquitous growth of indigenously isolated *Bacillus thuringiensis* isolate PKKVK 9 in Puducherry, Molasses favoured the growth of *Bacillus thuringiensis* at maximum (OD = 2.072) which was followed by Cassava Tippi (OD = 1.978), Luria Bertani Broth (OD = 1.745), Lignite (OD = 0.242) and the minimum growth was observed in Whey (OD = 0.228). Maximum biomass production of *Bacillus thuringiensis* was found in the substrate Luria Bertani broth (2.714 g/100ml) which was closer to the cell biomass production yielded by the substrate Molasses (2.619 g/100ml). The minimum cell biomass production was found in Whey (0.106 g/100ml). Of the substrate concentrations, 1% concentration was found to be optimum for the growth of *Bacillus thuringiensis* and also for maximum cell biomass and crystal protein production. The pH of the substrates played a crucial role in the growth as well as production of crystal protein by *Bacillus thuringiensis* effectively and among pH levels, pH 7 was found to be the optimum. Similarly, the substrate Molasses excelled the production of both cell protein (132.85µg/ml) and crystallaceous protein (32.63µg/ml) very well and the least cell and crystallaceous proteins levels (27.83 and 6.98µg/ml) were found with the substrate Whey. However, cell & crystallaceous proteins produced by synthetic substrate Luria Bertani Broth were nearing to Molasses while other substrates like Fishmeal, Cellulose, Soya peptone, Glucose, Sucrose also aided well in the production of cell and crystallaceous protein when compared to control.

INTRODUCTION

Ubiquitously present gram positive endospore forming bacterium *Bacillus thuringiensis* is known for producing crystalline inclusions containing one or more crystals called Pesticidal Crystal Proteins (PCPs) or δ-endotoxins during sporulation and thus killing the larvae of insect pests (Rajamohan *et al*., 1996 ; Dulmage and Rhodes, 1971 ; Goldberg *et al*., 1980 ; Beegle and Yamamoto,1992 and Navon, 2000). The crystals on dissolution in the insect midgut liberate protoxins which when proteolytically activated to a toxic fragment prove to be lethal to the larvae (Schnepf *et al*., 1998). In addition to PCPs, the bacterium capable of producing Vegetative Cell Proteins during the Vegetative growth phase. These are secreted proteins that also damage the insect midgut (Yu *et al*., 1997) and these toxins are considered to be environmentally safe. Several commercial preparations of *Bacillus thuringiensis* are of economically important when they use as microbial

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biopesticides for a number of crop pests (Sommerville *et al*., 1970; Sommerville and Pockett, 1975 and Shesser and Bulla, 1978). Formulation of *Bacillus thuringiensis* as bacterial biopesticide needs large quantities of spore crystal preparations with high insecticidal potency and this involves a pretty-penny. For large scale field application, efficient mass production techniques are necessary. Several techniques for the mass production of Entomopathogenic bacterium are mostly designed to yield infective colonies. These methods mostly vary on using different substrates as carbon and nitrogen sources, called media optimization (Yang and Wang, 2000).

Further, the low cost and ease of availability of agro-industry based wastes and organic substrates make them attractive for the production of *Bacillus thuringiensis* (Ampofo, 1995 and Prabagaran *et al.*, 2004). A simple and inexpensive method *viz*. one fed batch or continuous culture deviced by Rossa *et al*., (1992) yielded a sizable biomass and spore yield of *Bacillus thuringiensis* using the locally available natural as well as synthetic substrates. Hence, the production of Biopesticide *Bacillus thuringiensis* should be based on locally available materials to derive the desired economic advantage besides the knowledge on growth parameters and their interrelationship for maximum production of crystal proteins using either natural substrates or synthetic media. Hameed *et al.*, (1990) employed several media ingredients locally available in Egypt for their ability to eke-out endotoxin production for their native *Bacillus thuringiensis* strains. Similarly, screening of locally available raw material like Fishmeal, Molasses, Sago, Paddy straw and Whey, Molasses proved to be a good substrate among other locally available raw materials that could be used to support the enhanced biomass production of *Bacillus thuringiensis* strain PBT 372 in varying levels of inoculum, pH and substrate concentration (Prabagaran *et al*., 2004).

Bacillus thuringiensis was cultured in liquid media containing various commercial peptone sources to determine their effect, growth and colonization. It grew readily in synthetic media and was not exacting in its' nutritional requirements but tryptone, casein and yeast extract were good for not only production of endospores but also for bacterial colonial growth (Haridasan, 1991). Nutrient Agar, Polymedium, Katnelson's Tryptose Agar and Luria Bertani Agar could be used to culture *Bacillus thuringiensis* besides soya broth for high biomass production (Parks, 1993 and Anonymous, 1998). Yu *et al*., (1997) had modified the marscent production technique for culturing *Bacillus thuringiensis* wherein Vip 3 could be used for the control of insect pests. While Prabagaran *et al*. (2004) found suitability of Luria Bertani broth with 5 to 7 pH for culturing *Bacillus thuringiensis*, Vimaladevi *et al*., (2001) had developed a medium based on locally available cheap material wheat bran supplemented with organic salts for production of *Bacillus thuringiensis*. *Bacillus thuringiensis* sometimes do not grow on simple nutrient medium which contained glucose as the only carbon source while a medium containing yeast extract and soluble extract as main nitrogen and carbon sources and some organic basal salts substantiated *Bacillus thuringiensis* growth immensely (Faloci *et al*., 1990). Among 54 bacterial isolates obtained as of now, the most efficient *Bacillus thuringiensis* isolate PKKVK 9 capable of encountering insect pests by virtue of virulence, effectiveness and indigenous adaptability (Ramamourti *et al*., 2010). It is hence necessary to increase the biomass production of this *Bacillus thuringiensis* isolate using the locally available cheap raw materials by spill the beans namely "**Optimization of media**". Keeping this in view and availability of a dearth of information on utilizing the locally available materials for *Bacillus thuringiensis* production, the present study was aimed to optimize the locally available raw materials and synthetic substrates having carbon and nitrogen sources for increased production at low costs highly efficient *Bacillus thuringiensis* PKKVK 9 biopesticides for the control of crop pests.

MATERIALS AND METHODS

Slant culture of *Bacillus thuringiensis* PKKVK 9 was obtained from the repository culture collections of State Bio Control Laboratory, Perunthalaivar Kamaraj Krishi Vigyan Kendra, Puducherry.

Estimation of Growth and Biomass Production of *Bacillus thuringiensis* isolate PKKVK 9

The locally available raw materials and synthetic substrates like Glucose, Sucrose, Starch, Cellulose, Paddy Straw, Molasses, Skimmed Milk Powder, Whey, Sago, Cassava Tippi, Lignite and Charcoal having carbon source and the substrates like Soyamilk, Fish Meal, Soyapeptone, Bactopeptone, Wheat Bran having nitrogen source for maximum biomass production of *Bacillus thuringiensis* at various percentage levels *viz.*, 0.5, 1.0 and 1.5 were dissolved in 100 ml distilled water in 250 ml conical flasks with varying levels of pH *viz*., 5,7 and 9. The paddy straw was soaked in tap water for 3h. and filtered through country filter paper. Sago was boiled in tap water for 15 min. and filtered through cheese cloth. One hundred millilitres of the filtrates and substrates obtained were dispensed in 250 ml Erlenmeyer flask and basal salts were added as recommended by Faloci *et al*. (1990) and mixed well. Luria Bertani medium was used as reference medium. The Erlenmeyer flasks containing the medium were sterilized at 121^{o}C for 15 min. and allowed to cool. Overnight grown *Bt* a rotary shaker at 180 rpm at 30^{o}C for 72h. After 72h. of incubation, the growth of *Bt* in various substrates was measured as optical density at 600nm using Digital Bio Spectrophotometer. The grown cultures were centrifuged at 10,000 rpm for 1h. and the cell pellet was separated and weighed using the Electronic weighing balance and expressed as g / 100 ml of the substrate broth. Initial pH of the substrate and inoculum percent were chosen as independent variables whereas the growth of the culture and biomass values were treated as dependant variables. Three replications were maintained for each substrate.

Determination of Cell Protein in *Bacillus thuringiensis* isolate PKKVK 9

Proteins form complex with Copper ions in the alkali medium and this complex produces dark blue colour when

the aromatic amino acids like Tyrosine and Tryptophan present in the proteins reduce Phosphomolybdate and Tungstate of Folin - Phenol reagent. Hence, the Total Cell Protein was estimated using Folin-Ciocalteau method (Lowry *et al.*, 1951)

Ten millilitres of 72 h incubated indigenously isolated *Bacillus thuringiensis* PKKVK 9 isolate was taken in a 15 ml centrifuge tube and centrifuged at 10,000 rpm for 15 min. using High Speed Cooling Research Centrifuge. One millilitre of the centrifuged supernatant sample was transferred into a test tube (18 x 150 mm) and five millilitres of Alkaline Reagent (Solution A : Sodium Carbonate 1g, Sodium Hydroxide 0.2g and Monosterile Distilled Water 50 ml ; Solution B : Sodium Potassium Tartarate 200mg and Monosterile Distilled Water 10ml ; Solution C : Copper Sulphate 100mg and Monosterile Distilled Water 10ml and the solution A, B and C are mixed in the ratio of 100 : 1 : 1) and 0.5 ml of Folin's Ciocalteau's Reagent were added and incubated in dark for 30 min. Colour developed in the protein sample at the end was read at 660 nm using Bio Spectrophotometer and the total Cell Protein present in *Bacillus thuringiensis* PKKVK 9 isolate was estimated and expressed as µg/ml. The Bovine Serum Albumin with different concentrations *viz.*, 20, 40, 60, 80 and 100 µg were used as Standard. Three replications were maintained.

Estimation of Crystallaceous Protein in *Bacillus thuringiensis* isolate PKKVK 9

Preparation of spores crystal mixture

Spore suspension of indigenously isolated *Bacillus thuringiensis* bacterial isolate PKKVK 9 maintained in T3 Agar medium (Tryptone 3g, Tryptose 3g, Yeast Extract 1.5g, Sodium Phosphate cultures were inoculated individually and incubated in (9 cm diameter) containing T3 Agar Medium under aseptic conditions and the plates were incubated at 30°C for overnight. The overnight grown *Bacillus thuringiensis* culture was inoculated into 30 ml T_3 broth in 250 ml conical flask and incubated at 30°C under shake culture conditions. The bacterial sporulation was monitored carefully when more than ninety percent lysis was reached and the sporulated culture containing both spores and crystals in conical flask was transferred to 4°C atleast half an hour before harvesting.

The spore crystal mixture of the bacterial isolate was scrapped as flakes and transferred to conical flask containing icecold 2.5 ml Tris Ethylene Diamine Tetrachloro Acetic Acid (EDTA) buffer (Tris 10 mM, EDTA 1 mM, pH 8) and 1 mM Phenyl Methyl Sulphonyl Fluoride (PMSF). The contents in conical flask was centrifuged at 10000 rpm for 10 min. at 4°C and pellet was obtained. The pellet was washed once with 10 ml ice cold 0.5 mM NaCl at 10000 rpm for 10 min. followed by two washes using 10 ml Tris – EDTA buffer at the same speed and time. Finally, the spore crystal pellet was suspended in one millilitre Sterile Distilled Water with 1 mM PMSF. The above said operations were carried out at 4°C in order to prevent possible degradation of crystal proteins.

Detection of Crystalleous Protein

The crude spore crystal mixture of *Bacillus thuringiensis* isolate PKKVK 9 was centrifuged at 10000 rpm for 10 min. at 4°C and 300 µl of Solubilizing Buffer (50 mM Na_2CO_3, 10 mM Dithiothreitol, pH 10.5) was added with 25mg wet weight of spore crystal pellet. This was mixed by vortexing and incubated at 37°C under slow shaking for 4 h. After incubation, the sample was again centrifuged at 10000 rpm for 10 min. at 4°C. The content was filtered through Whatman No. 1 filter paper and the supernatant containing the solubilized crystal protein free from spores and undissolved inclusions was used for the estimation of crystallaceous protein by Bradford's method (Bradford, 1976). Three replications were maintained.

RESULTS AND DISCUSSION

With view to find out alternative cheap raw materials for production of *Bacillus thuringiensis* biopesticide, naturally occurring raw materials like Glucose, Sucrose, Starch, Cellulose, Paddy Straw, Molasses, Skimmed Milk Powder, Whey, Sago, Cassava Tippi, Lignite and Charcoal having carbon source and the substrates like Soyamilk, Fish Meal, Soyapeptone, Bactopeptone, Wheat Bran having 0.05M, MnCl 0.005M, Agar 20.0 g and Sterilized Mono Distilled Water 1litre) was spread on petriplates and this was compared with those using Luria Bertani broth as reference medium and the findings are presented in Table - 1. It is clear from the data that the growth and biomass production of *Bacillus thuringiensis* varied among themselves individually with respect to varying levels of pH and substrate concentrations. Similarly, the substrates tested irrespective of Carbon and Nitrogen sources also varied significantly. Molasses favoured the growth of *Bacillus thuringiensis* at maximum (OD = 2.072) which was followed by Cassava Tippi (OD = 1.978), Luria Bertani Broth (OD = 1.745), Lignite (OD = 0.242) and the minimum growth was observed in Whey (OD = 0.228). (Fig. 1) Maximum biomass production of *Bacillus thuringiensis* was found in the substrate Luria Bertani broth (2.714 g/100ml) which was closer to the biomass production yielded by the substrate Molasses (2.619 g/100ml). The minimum biomass production was found witnessed in Whey (0.106 g/100ml). (Fig. 2) The other substrates intermediary followed suit. The growth of the bacterium *Bacillus thuringiensis* as well as biomass production are directly proportional to the level of substrate concentrations and among the substrate concentrations 1% concentration was found to be optimum for the growth of *Bacillus thuringiensis* and also for maximum cell biomass production. The pH of the substrates played a crucial role in the growth as well as production of biomass of *Bacillus thuringiensis* effectively and all the pH levels *viz.*, 5, 7 and 9 varied significantly. Of the pH levels, pH 5 invariably exhibited the minimum level of *Bacillus thuringiensis* growth and biomass production. There was a striking increase in the growth and biomass production of *Bacillus thuringiensis* in pH 7 when compared to pH 5 irrespect of the substrates tested in the study. Besides this, a slight decline of bacterial growth in pH 9 was noticed because of alkalinity. Among pH

Table 1 Suitability of certain natural and synthetic substrates for growth and cell biomass production of *Bacillus thuringiensis* isolate PKKVK 9

Substrate	Substrate concentration (%)	Optical Density Value* at pH Level			Mean	Cell Biomass* (g / 100 ml) at pH Level			Mean
		5	**7**	**9**		**5**	**7**	**9**	
Glucose	0.5	0.581	0.707	0.222	0.503	0.917	1.600	0.967	1.161
	1.0	0.833	1.075	0.411	0.773	0.936	1.627	1.990	1.517
	1.5	0.760	1.136	0.412	0.769	0.987	2.001	1.817	1.601
Sucrose	0.5	0.153	0.363	0.518	0.344	0.519	1.880	1.070	1.156
	1.0	0.255	0.842	0.486	0.527	0.870	1.529	1.017	1.138
	1.5	0.234	0.880	0.596	0.570	0.839	1.815	1.817	1.490
Starch	0.5	0.494	0.768	0.263	0.508	0.827	1.143	0.861	0.943
	1.0	0.487	0.932	0.992	0.803	1.515	1.005	1.439	1.319
	1.5	0.610	1.009	1.081	0.900	1.099	1.323	1.316	1.246
Cellulose	0.5	0.761	1.416	0.785	0.987	1.295	2.994	1.299	1.862
	1.0	1.199	1.257	0.950	1.135	1.675	1.667	1.714	1.685
	1.5	1.602	1.762	1.465	1.609	1.751	1.869	1.826	1.815
Paddy Straw	0.5	0.817	0.185	0.048	0.350	1.269	0.902	0.389	0.853
	1.0	1.082	1.235	0.285	0.867	1.625	0.850	1.039	1.171
	1.5	0.420	0.967	0.473	0.620	1.334	0.875	1.119	1.109
Molasses	0.5	0.981	1.829	0.798	1.202	2.300	2.999	2.162	2.487
	1.0	2.032	2.658	1.526	2.072	2.505	2.619	1.919	2.347
	1.5	1.686	2.110	1.804	1.866	2.756	2.618	2.483	2.619
Skimmed Milk Powder	0.5	0.386	0.382	0.317	0.361	0.770	0.747	0.637	0.718
	1.0	0.428	0.720	0.486	0.544	0.898	0.823	1.200	0.973
	1.5	0.331	0.614	0.056	0.333	1.264	1.806	2.003	1.691
Whey	0.5	0.139	0.261	0.285	0.228	0.087	0.016	0.215	0.106
	1.0	0.321	0.317	0.265	0.301	1.093	1.017	1.013	1.041
	1.5	0.428	0.423	0.418	0.423	1.750	1.987	1.119	1.618
Sago	0.5	0.758	0.774	0.538	0.690	1.415	1.470	1.005	1.296
	1.0	0.961	2.075	0.864	1.300	1.792	1.651	1.088	1.510
	1.5	0.938	2.047	0.977	1.320	1.450	1.784	1.817	1.683
Cassava Tippi	0.5	0.693	0.946	0.279	0.639	0.810	1.534	0.977	1.107
	1.0	1.233	1.768	1.073	1.358	1.996	1.269	1.390	1.551
	1.5	1.538	2.376	2.022	1.978	1.292	2.329	2.934	2.185
Lignite	0.5	0.188	0.343	0.196	0.242	0.826	0.763	1.273	0.954
	1.0	0.286	0.529	0.548	0.454	0.841	1.777	0.841	1.153
	1.5	0.219	0.376	0.469	0.354	1.741	1.711	1.253	1.568
Charcoal	0.5	0.650	1.421	0.807	0.959	1.036	1.474	1.676	1.395
	1.0	0.820	1.629	0.918	1.122	0.544	1.507	2.060	1.370
	1.5	0.449	1.603	0.900	0.984	0.957	1.477	1.696	1.376
Soyamilk	0.5	0.442	0.879	0.487	0.602	1.910	0.542	1.016	1.156
	1.0	0.483	0.937	0.466	0.628	1.435	1.627	1.857	1.639
	1.5	0.596	0.527	0.505	0.542	1.544	1.558	1.673	1.591
Fishmeal	0.5	0.678	1.524	0.989	1.063	2.186	1.916	2.018	2.040
	1.0	0.719	1.820	0.999	1.179	2.435	2.316	2.518	2.423

	1.5	0.761	1.513	1.000	1.091	2.658	2.312	2.160	2.376
Soyapeptone	0.5	0.326	1.106	0.617	0.683	0.869	0.913	0.716	0.832
	1.0	0.483	0.919	0.713	0.705	1.023	1.009	0.968	1.000
	1.5	0.461	0.942	0.723	0.708	1.161	1.132	0.991	1.094
Bactopeptone	0.5	0.238	0.939	0.615	0.597	0.763	0.814	0.714	0.763
	1.0	0.348	1.129	0.713	0.730	0.813	0.865	0.816	0.831
	1.5	0.366	1.213	0.816	0.798	0.917	0.865	0.923	0.901
Wheat bran	0.5	0.234	0.811	0.368	0.471	0.832	0.816	0.653	0.767
	1.0	0.299	0.816	0.413	0.509	0.916	0.932	0.769	0.872
	1.5	0.336	0.821	0.493	0.550	0.813	0.863	0.635	0.770
Luria Bertani Broth	0.5	1.126	1.677	0.959	1.254	2.377	2.617	1.980	2.324
	1.0	1.878	1.964	1.394	1.745	2.642	2.754	2.746	**2.714**
	1.5	1.770	1.720	1.590	1.693	2.615	2.499	2.542	2.552
Control	0.5								
	1.0	0.423	0.423	0.423	0.423	0.160	0.160	0.160	**0.160**
	1.5								

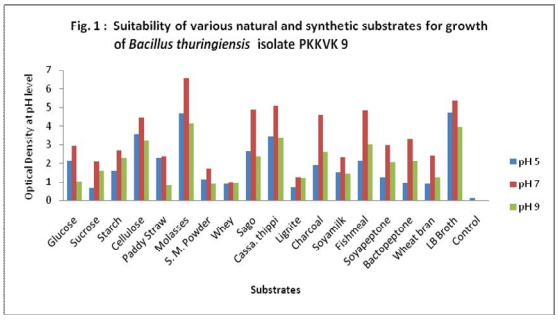
Interaction between substrates and concentrations

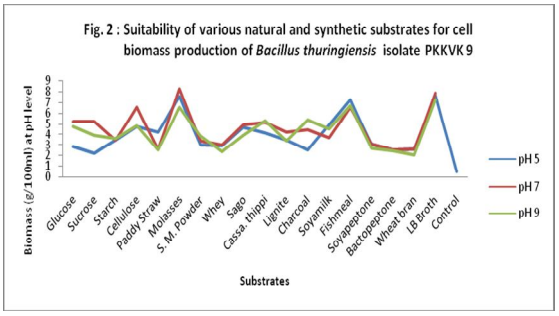
C.D (p=0.05)	0.0547	0.1320	0.0466	0.0764	0.0734	0.0101
S.Ed.	0.0279	0.0677	0.0238	0.0390	0.0375	0.0052

Interaction between substrates and pH Levels

Interaction level	Level of Significance	Significance		S.Ed.		C.D	
		OD	**Biomass**	**OD**	**Biomass**	**OD**	**Biomass**
Between pH	0.05	**	**	0.4639	0.5932	0.9234	1.1192
Between substrates	0.05	**	**	0.1917	0.2907	0.3757	0.5698
Between pH and substrates	0.05	**	**	0.1178	0.1448	0.2308	0.2839

*Each value is a mean of three replications
Means with same alphabet do not differ significantly
Data analyzed by two factorial RBD using square root transformed values and ranking bolded by DMRT





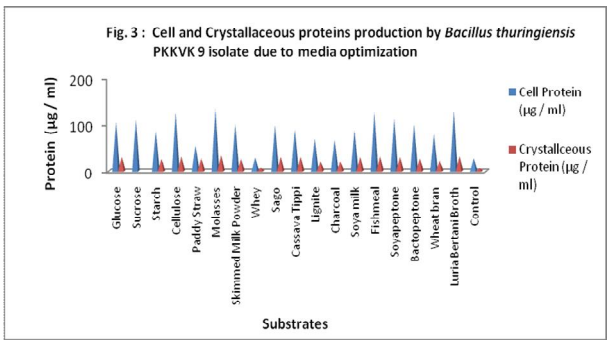
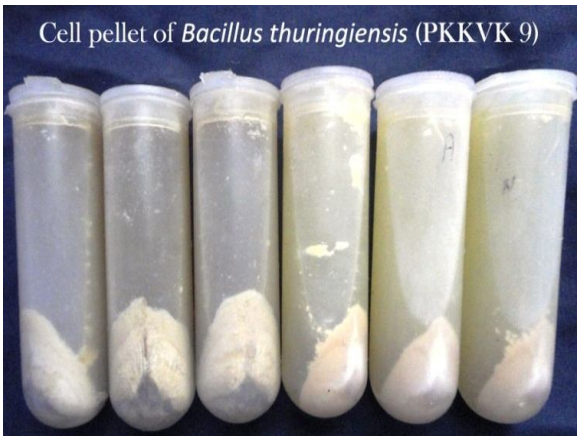
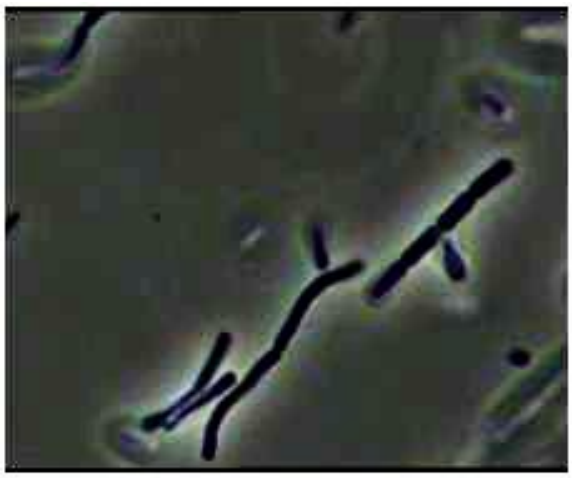


Table 2 Cell and Crystallaceous Proteins yield of *Bacillus thuringiensis* PKKVK 9 isolate due to optimization of certain natural and synthetic substrates

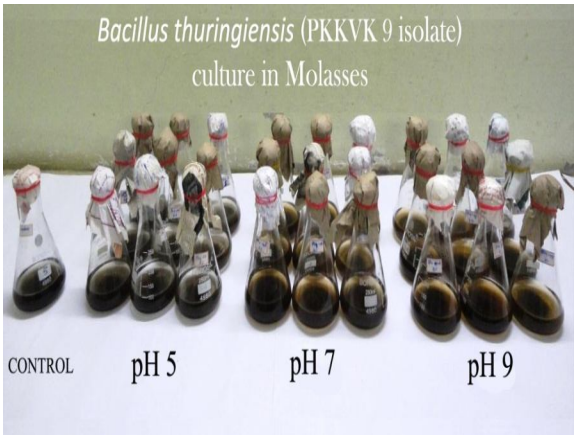
Sl. No.	Substrate	Cell Protein* (µg / ml)	Crystallceous Protein* (µg / ml)
1.	Glucose	105.42^{e-l} (10.26)	29.36^{b-e} (5.41)
2.	Sucrose	109.94^{d-l} (10.48)	29.96^{b-e} (5.47)
3.	Starch	85.36^{h-l} (9.23)	25.04^{cde} (5.00)
4.	Cellulose	124.96^{c-l} (11.17)	29.85^{b-e} (5.46)
5.	Paddy Straw	54.96^{kl} (7.41)	26.04^{cde} (5.10)
6.	Molasses	**132.85^{a-l}** (11.52)	**32.63^{a-e}** (5.71)
7.	Skimmed Milk Powder	99.85^{f-l} (9.99)	25.09^{cde} (5.00)
8.	Whey	**27.83^{l}** (5.27)	**6.98^{e}** (2.64)
9.	Sago	98.27^{f-l} (9.91)	29.33^{b-e} (5.41)
10.	Cassava Tippi	89.09^{g-l} (9.43)	29.35^{b-e} (5.41)
11.	Lignite	69.05^{jkl} (8.30)	19.38^{de} (4.40)
12.	Charcoal	66.71^{jkl} (8.16)	20.09^{de} (4.48)
13.	Soya milk	87.38^{g-l} (9.34)	29.28^{b-e} (5.41)
14.	Fishmeal	125.09^{c-l} (11.18)	29.99^{b-e} (5.47)
15.	Soyapeptone	112.27^{d-l} (10.59)	29.38^{b-e} (5.42)
16.	Bactopeptone	99.98^{f-l} (9.99)	26.21^{cde} (5.11)
17.	Wheat bran	78.29^{i-l} (8.84)	20.85^{de} (4.56)
18.	Luria Bertani Broth	128.96^{b-l} (11.35)	30.61^{b-e} (5.53)
19.	Control	26.08^{l} (5.10)	6.25^{e} (2.50)
	C.D (p = 0.05)	**2.9696**	**1.8465**
	S. Ed.	**1.4774**	**0.9187**

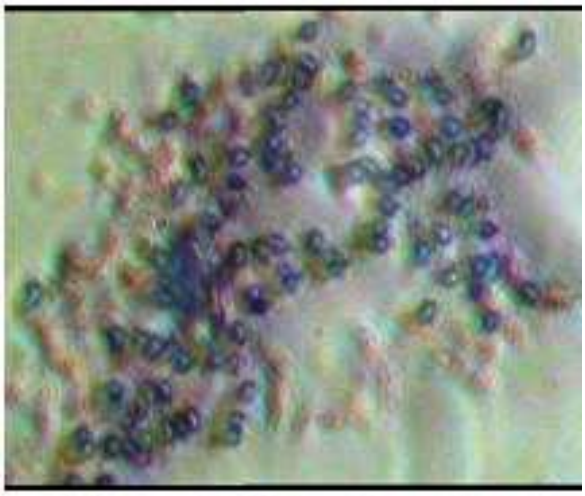
*Each value is a mean of three replications
Figures in parentheses are square root transformed values
Means with same alphabet do not differ significantly by DMRT





Rod Shape Vegetative Cells of *Bacillus thuringiensis* isolate PKKVK 9





Bipyramidal Shape Crystals of *Bacillus thuringiensis* isolate PKKVK 9

levels, pH 7 was found to be optimum for the growth and biomass production of *Bacillus thuringiensis.*

The study gains support from Prabagaran *et al*., (2004) who opined that the large scale production of *Bacillus thuringiensis* PBT – 372 should be based on their availability, cost and ease with which the bacterium utilizes their substrates. They found that the growth of *Bacillus thuringiensis* was more in media containing Luria Bertani broth followed by Fishmeal and maximum cell protein production in Molasses substrate. Present investigation sought support from the study carried out by Vimaladevi *et al.*, (2001) who obtained the significant level of *Bacillus thuringiensis* spores by using Wheat bran supplemented with basal nitrogen source and the fish meal as combination of carbon and nitrogen sources were used to grow *Bacillus thuringiensis* under shake culture conditions up of *Bacillus thuringiensis* production using basal salts in batch cultures technique by Faloci *et al*., (1990) and Rossa *et al*., (1992). The findings of present study also corroborates the enormous toxin protein production of *Bacillus thuringiensis* when optimizing certain natural as well as synthetic media by Yang and Wang (2000).

Results pertaining to yield of total Cell as well as Crystallaceous Proteins by *Bacillus thuringiensis* PKKVK 9 isolate due to optimization of certain natural and synthetic substrates are given (Table-2). From the Table, it is clear that the substrate, Molasses notched maximum quantities of both Cell protein (132.85 µg/ml) and Crystallaceous protein (32.63 µg/ml) while the minimum quantities of cell protein (27.83 µg/ml) and crystallaceous proteins (6.98 µg/ml) were found in the substrate, Whey. However, Synthetic substrate Luria Bertani Broth produced 128.96 µg/ml cell protein and 30.61 µg/ml crystallaceous protein which were inching to the proteins produced by the substrate Molasses. Further, it is distinct that the substrates like Fishmeal, Cellulose, Soyapeptone, Sucrose and Glucose had aided well in the production of cell and crystallaceous proteins when compared to Control. (Fig. 3) On the whole, Molasses excelled in the production of cell and crystallaceous protein of *Bacillus thuringiensis* PKKVK 9 isolate and other substrates followed suit. The findings gain support from similar results obtained by Prabagaran, 2000 and Prabagaran, *et al*., 2004 wherein they found Molasses which supported the maximum production of cell protein (65 µg/ml) and whey had a minimum of 59 µg/ml cell protein among the substrates they screened.

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