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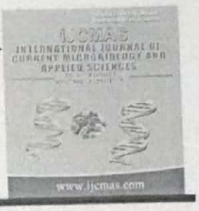

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Original Research Article

Optimization of Bacitracin Production from *Bacillus licheniformis* NCIM 2536

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Antibiotic production, Bacitracin, Wheat bran, Rice hulls, Soyabean meal

ABSTRACT

Bacillus licheniformis produces mainly polypeptide antibiotic such as bacitracin. The cheap raw materials used for its production were wheat bran, rice hulls and soyabean meal. The present study is concerned with the biosynthesis and optimization of antibiotic bacitracin by *Bacillus licheniformis* on laboratory scale. All the three raw materials taken for the study like wheat bran, soya bean meal and rice hulls demonstrated the production of Bacitracin efficiently. The 40^0C was the optimum temperature for bacitracin production from all the raw materials used and was found to be antibacterial against *Staphylococcus aureus*. In average the optimum pH for bacitracin production was in the range of 7 and 9 for wheat bran and soya bean meal respectively. Whereas, for rice hulls pH 4 was optimum for bacitracin production. The optimum time for bacitracin production was found to be 24 hrs.

Introduction

Antibiotics are low molecular-weight molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. A natural assumption is that soil microbes produce antibiotics in their natural habitat and use them to gain advantage over their competitors (Katz and Demain, 1977). Of the several hundred naturally produced antibiotics that have been purified, only a few have been sufficiently non-toxic to be of use in medical practice. Regardless of the toxicity of some antibiotics produced by bacteria from *Bacillus* genus to the cells of mammals (e.g. polymyxins, bacitracin, etc.), they were and continued to be in the focus of attention of scientists. In pharmaceutical industry, several peptide antibiotics of importance are produced by *Bacillus* species such as bacitracin, polymyxin, gramicidin, tyrocidine, subtilin, bacilysin etc (O'Grady and Greenwood, 1997; Trookman et al., 2011).

Peptide antibiotics form a unique group of bioactive molecules (Schrooder, 1999). Most of the peptide antibiotics produced by *Bacillus* are active against gram positive bacteria (Ming and Epperson, 2002). However, compounds such as polymyxin,

colistin and circulin exhibit activity almost exclusively upon gram-negative forms, whereas bacillomycin, mycobacillin and fungistatin are effective agents against molds and yeasts. Bacitracin consists of one or more of the antimicrobial polypeptides or cyclic polypeptides produced by certain strains of *Bacillus licheniformis* and by *Bacillus subtilis* (Reiko et al., 2003).

Different types of bacitracin like A, A1, B, C, D, E, F, F1, F2, F3 and G have been discovered. The most potent antibiotic is Bacitracin A, whereas Bacitracin B and C are less potent and the rest posses very little antibacterial activity. This antibiotic is most effective against Gram positive (+ve) and a few Gram negative (-ve) species of bacteria. It is almost exclusively used as a topical preparation in the treatment of infections (Brunner et al., 1965). Bacitracin is used in human medicine as a polypeptide antibiotic as bacitracin zinc salt, in combination with other topical antibiotics (usually polymyxin B and neomycin) as an ointment ("triple antibiotic ointment," with a common brand name Neosporin), used for topical treatment of a variety of localized skin and eye infections, as well as for the prevention of wound infections. A non-ointment form of ophthalmic solution is also available for eye infections. Although allergic cross reaction with sulfa drugs has been occasionally reported, bacitracin-containing topical preparations remain a possible alternative to silver sulfadiazine (Silvadene) for burn patients with a sulfa allergy (Hammes and Frank, 1979).

In one of study, the soil sample was collected and screened for isolation of higher antibiotic producing bacterial strain. The isolated bacterial strain was tentatively identified as *Bacillus* spp. by biochemical characteristics using Bergey's manual. Their study was focused on the optimization of different parameters for production of peptide antibiotic (Bacitracin) using synthetic medium. The antibacterial activity of isolate was analyzed against *S. aureus*. The nutritional ingredients for bacitracin production were found as glucose (1%) and L-glutamic acid (0.5 %) as carbon and nitrogen source respectively. The prominent bacitracin production was obtained after 48 hrs incubation at 40°C temperature and at 7 pH (Weston et al., 2001).

Bacillus licheniformis is a bacitracin producing microorganism. Bacitracin consists of one or more of the antimicrobial polypeptides produced by certain strains of *Bacillus licheniformis*. The cheap raw material for bacitracin production should be readily available and cheap such as wheat bran, rice hulls, soyabean meal, etc. Thus development of this technology in our country would result in saving reasonable amount of foreign exchange by exploiting indigenous resources. The aim of the present study was to optimize the production of bacitracin by using *Bacillus licheniformis* NCIM 2536 from the cheap raw material such as wheat bran, rice hulls and soyabean meal.

Materials and Methods

Collection of Bacteria: The bacteria *Bacillus licheniformis* NCIM 2536 was obtained from National Chemical Laboratory (NCL) Pune. *Bacillus licheniformis* was used for the production of antibiotic bacitracin. The culture was maintained on tryptone glucose yeast extract agar medium for further use.

Collection of Raw Materials: Raw materials such as wheat bran, rice hulls and soyabean meal were used for bacitracin production.

Inoculum Preparation: The Basal medium was first prepared by mixing 10.0 g peptone, 5.0 g glucose, 5.0 g beef extract, 2.5 g sodium chloride, 0.167 g $MnCl_2$, 1000 ml distilled water and final pH 7.0. The bacterial growth was aseptically scrapped from 48 hours old agar slants and transferred to 50 ml sterilized basal medium in 250 ml conical flask and incubated on rotary shaker incubator at 150 rpm for 24 hours at 37^0C. The vegetative culture obtained was used for inoculation into fermentation medium.

Bacitracin Production: Fermentation media was used for the production of antibiotic bacitracin by *Bacillus licheniformis*. Fermentation media was prepared by mixing 1.0 g citric acid, 0.5 g glucose, 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.01 g $MnSO_4.4H_2O$, 0.5 g K_2HPO_4, 0.01g $FeSO_4.7H_2O$, 1000ml distilled water and pH was maintained at 7.0. In this way three flasks having fermentation medium were prepared. In these flasks the three raw materials like wheat bran, rice hulls and soyabean meal were added separately (45 g/1000ml). The media were sterilized at 15 lbs pressure, (121^0C) for 15 minutes. The media were inoculated with 1 ml seed culture. After inoculation, shake flask fermentation medium (incubated at 37^0C at 150 rpm for 96 hours) was used for antibiotic production. At the end of the fermentation period, the fermented material was soaked in N/100 HCl for 1 hour. It was centrifuged at 10000 rpm for 20 minutes at 4^0C to get cell free supernatant. The pellet was discarded and sterilized supernatant was used for agar well diffusion assay.

Optimization of Bacitracin Production:

Bacitracin production from different raw materials was carried out by applying various parameters. The parameters include pH (4, 7, 9), temperature (28^0C, 37^0C, 40^0C) and time (24 hrs 48 hrs, 72 hrs, 96 hrs) (Bushra et al., 2006).

Extraction and Detection of Bacitracin:

For extraction of Bacitracin, 1 ml of the fermented medium from varying temperature, pH and incubation time, was taken. The supernatant was extracted twice with n-butanol and ether (50% n-butanol and 50% ether) in a separating funnel using half the solvent for each extraction. Concentrated hydrochloric acid was added in drops along the sides of the wall until it separates into two layers. Bacitracin was found in the lower layer. The filtrate was separated by filtration using Whatman filter paper. The extracted bacitracin was identified by paper chromatography. Commercially available purified bacitracin was used as a standard. The ascending chromatography was carried out on Whatman paper No. 1. The solvents used were Acetone, Acetic acid and H_2O at 20:06:74 ratios. The paper was dried and the spots were made visible by spraying ninhydrin. The Rf value for the standard antibiotic was calculated. This value was then matched with that of the antibiotic obtained by performing various parameters for its confirmation as the Bacitracin (Bushra et al., 2006).

Assay of Bacitracin: The activity of the antibiotic bacitracin present in the fermented material was determined by agar well diffusion method (Harvel et al., 1999). Nutrient agar plates were prepared and the lawn of 24 hrs old broth culture of *Staphylococcus aureus* NCIM 2079 was prepared by using sterile cotton swab. By using sterile cork borer having diameter 6mm, the wells were prepared in the nutrient agar plates. A 0.5 ml of bacitracin was added in the well. The plates were incubated for 24 hours at 37^0C. After incubation the zone of inhibition was observed.

Results and Discussion

Three different cheap raw materials such as wheat bran, rice hulls and soya bean meal were used for bacitracin production by using *Bacillus licheniformis* NCIM 2536. The different parameters were also applied for studying the bacitracin production in terms of zone of inhibition observed on the plate lawn with *Staphylococcus aureus*. According to Montserrat et al., (2000), the production of the antibiotic, however, was found to be maximum in the presence of soybean meal and sucrose or mannose. After performing the paper chromatography the Rf value of standard Bacitracin was found to be 0.9. Matching to this the crude antibiotic also given the same Rf value. Thus on the basis of migration, the crude antibiotic was found to be the bacitracin. In the present study the Bacitracin was extracted successfully (Andrea et al., 2001; Reiko et al., 2003). The antibiotic produced in present study was confirmed to be bacitracin as it inhibits the Gram positive bacteria, and also confirmed to be bacitracin by paper chromatography. The same results were achieved by Snell et al., (1955).

At varying temperature and at incubation period of 24hrs, it was observed that at 28^0C there was zone of inhibition in only soya bean meal (12 mm) while at 37^0C zone of inhibition in wheat bran (20 mm) and soya bean meal (19 mm); at 40^0C zone of inhibition in wheat bran (12 mm) (Figure 3). Thus maximum antibiotic production was found at 37^0C after 24 hrs of incubation by using Wheat bran and Soya bean meal (Table 1).

At varying temperature and at incubation period of 48 hrs it was observed that at 28^0C there was no zone of inhibition while at 37^0C wheat bran and rice hulls show 14 mm and 12 mm zone of inhibition respectively (Figure 2) whereas at 40^0C zone of inhibition was observed for all the substrates like wheat bran (22 mm), rice hulls (20 mm) and soyabean meal (24 mm). Maximum antibiotic production at 40^0C after 48 hrs of incubation was obtained from soya bean meal followed by wheat bran and rice hulls (Table 2) (Graph 1) (Figure 1).

At varying temperature and at incubation period of 72 hrs it was observed that at 28^0C there was zone of inhibition observed only in wheat bran (14 mm) whereas at 37^0C zone of inhibition in soya bean meal (13 mm); at 40^0C zone of inhibition in soya bean meal (14 mm). Significant antibiotic production was observed at 28^0C (wheat bran) and at 40^0C (soya bean meal) after 72 hrs of incubation (Table 3).

At varying temperature and at incubation period of 96 hrs it was observed that at 28^0C there was no zone of inhibition whereas at 37^0C zone of inhibition in rice hulls (12 mm) and soya bean meal (13 mm); at 40^0C zone of inhibition in wheat bran (14 mm), rice hulls (16 mm) and soya bean meal (16 mm). Antibiotic production was observed significantly at 40^0C after 96 hrs of incubation (Table 4). In the present study 40^0C was the optimum temperature for bacitracin production. Temperature is also an important regulator of the rate of metabolism and the growth rate of microorganisms. Hunt and Steiber (1986) reported that a shift in temperature can alter the utilization rate of one component as compared to another, thus unbalancing the medium with respect to growth. In the study of Anker et al., (1947) temperature optimization was also carried out and it was found that antibiotic formation was dependent on temperature. According to Egorov et al., (1983) it was concluded that the effect of temperature ranging from 30^0C to 55^0C antibiotic production was maximum

at 37^{0}C by using *Bacillus licheniformis*. Their study suggested that the synthesis of bacitracin was substantially sensitive to the temperature variation.

At varying pH and at incubation period of 48 hrs it was observed that at acidic 4 pH there was no zone of inhibition where as at neutral 7 pH zone of inhibition in wheat bran (20 mm); at alkaline 9 pH no zone of inhibition. At pH 7, after 24 hrs, the maximum antibiotic was produced (Table 5) (Graph 2).

At varying pH and at incubation period of 48 hrs it was observed that at acidic 4 pH there was no zone of inhibition in soya bean meal and wheat bran but rice hulls showed 14 mm of zone of inhibition. Whereas at neutral pH 7 zone of inhibition was observed for all the substrates like wheat bran (14 mm), rice hulls (12 mm) and soyabean meal (16 mm). Also at alkaline pH 9 the zone of inhibition was observed for wheat bran (20 mm) and rice hulls (12 mm) but no zone of inhibition in soyabean meal. After 48 hrs of incubation, maximum antibiotic was produced at pH 9 (Table 6) (Graph 2).

At varying pH and at incubation period of 72 hrs it was observed that at acidic 4 pH there was zone of inhibition in rice hulls (12 mm) and soya bean meal (13 mm) whereas at neutral 7 pH zone of inhibition in soybean meal (13 mm). At alkaline 9 pH zone of inhibition in wheat bran (18 mm) was observed. At pH 9.0, maximum antibiotic was produced from wheat bran after 72 hrs of incubation (Table 7).

At varying pH and at incubation period of 96 hrs it was observed that at acidic 4 pH there was zone of inhibition in wheat bran (16 mm) and soyabean meal (12 mm), at pH 7 in rice hulls (12 mm) and soyabean meal (13 mm). At alkaline 9 pH no zone of inhibition was observed. After 48 hrs of incubation significant antibiotic was produced from wheat bran, at pH 4 (Table 8). Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites. Bacitracin production by *Bacillus licheniformis* is pH dependent reported by Montserrat et al., (2000). In the present study pH was also optimized, during optimization of pH it was observed that best activity was achieved at pH range of 7-9 with maximum activity at pH 9. The same results were achieved by Snoke (1960); Hanlon and Hodges (1981), where the formation of bacitracin by *B. licheniformis* was maximum at pH 8.

At varying time it was observed that at 24 hrs there was zone of inhibition in wheat bran (20 mm) and soyaben meal (19 mm) but no zone of inhibition in rice hulls. Whereas at 48 hrs, zone of inhibition was observed in wheat bran (22 mm) and rice hulls (12 mm) but no zone of inhibition in soyabean meal. At 72 hrs zone of inhibition was observed only in soyabean meal (13 mm) and no zone of inhibition in wheat bran and rice hulls. At 96 hrs zone of inhibition was observed in rice hulls (12 mm) and soyabean meal (13 mm). After 24 hrs of incubation maximum antibiotic was produced from wheat bran (Table 9) (Graph 3). The similar result was found by Awais et al., (2008) at varying incubation period the maximum zone of inhibition was observed in case of *B.pumilus* against *S. aureus* (19mm) and *M. luteus* (17mm) after 24 hours. On the other hand after 48, 72, 96, 120 and 144 hours a gradual decrease in activity was seen against *S. aureus* and *M. luteus*. Whereas *B. subtilis* showed no activity against *S. aureus*, while against *M. luteus* maximum activity was detected (14mm), followed by a gradual decrease with time.

In the present study at 24 hours maximum bacitracin production takes place. This result was found to be contradictory with that of Demain (1972), according to their study, incubation time optimization given 96 hours as optimum time for antibiotic production. As antibiotics are secondary metabolites so these are formed usually when organism has passed rapid growth phase. The same thing was discussed by Demain. He concluded that the synthesis of peptide antibiotics is initiated after the organism has passed the rapid growth phase. Although antibiotic formation usually follows logarithmic growth (presumably due to some type of repression of antibiotic synthatases in the growth phase), this is not universally observed. It is clear from the study of Hanlon and Hodges (1981) that antibiotics are sometimes produced during growth and that both genetic and nutritional modifications can shift the time of antibiotic synthesis in relation to the growth phase and concluded that bacteria under certain conditions, arise throughout the phase of growth which is truly exponential rather than the phase of active but non exponential growth. Thus further increase in fermentation period resulted in the decline of bacitracin activity. It may be attributed to inhibition of "Bacitracin synthetase" enzyme by bacitracin itself by feedback inhibition Farzana et al., (2004).

Thus, bacitracin is the important antibiotic which can be produced from cheap raw material like rice hulls, wheat bran and soya bean meal. In the present study all the three raw materials have given the production of bacitracin therefore the better yield of bacitracin might be due to the porosity of the substrate. This is because the supply of oxygen to the culture resulted in greater antibiotic yield. The results were in the context of antibiotic production with that of the results of Farzana et al., (2004).

Table.1 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soya bean meal at Varying Temperature (Incubation Time 24 hours)

Substrate	28^0C	O.D.	37^0C	O.D.	40^0C	O.D.
Wheat bran	-	0.28	20 mm	0.38	12 mm	0.27
Rice hulls	-	0.08	-	0.09	-	0.08
Soya bean meal	12 mm	0.19	19 mm	0.16	-	0.38

Table.2 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soya bean meal at Varying Temperature (Incubation Time 48 hours)

Substrate	28^0C	O.D.	37^0C	O.D.	40^0C	O.D.
Wheat bran	-	0.05	14 mm	0.22	22 mm	0.01
Rice hulls	-	0.12	12 mm	0.08	20 mm	0.26
Soya bean meal	-	0.06	-	0.17	24 mm	0.01

Table.3 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soya bean meal at Varying Temperature (Incubation Time 72 hours)

Substrate	28^0C	O.D.	37^0C	O.D.	40^0C	O.D.
Wheat bran	14 mm	0.04	-	0.36	-	0.02
Rice hulls	-	0.08	-	0.01	-	0.10
Soya bean meal	-	0.12	13 mm	0.10	14 mm	0.02

Table.4 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soya bean meal at Varying Temperature (Incubation Time 96 hours)

Substrate	28^0C	O.D.	37^0C	O.D.	40^0C	O.D.
Wheat bran	-	0.01	-	0.06	14 mm	0.02
Rice hulls	-	0.16	12 mm	0.06	16 mm	0.16
Soya bean meal	-	0.09	13 mm	0.15	16 mm	0.05

Table.5 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soyabean meal at Varying pH (Incubation Period 24 hours)

Substrate	pH 4.0	O.D.	pH 7.0	O.D.	pH 9.0	O.D.
Wheat bran	-	0.04	20 mm	o.38	-	0.02
Rice hulls	-	0.06	-	0.09	-	0.03
Soya bean meal	-	0.10	-	0.16	-	0.02

Table.6 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soyabean meal at Varying pH (Incubation Period 48 hours)

Substrate	pH 4.0	O.D.	pH 7.0	O.D.	pH 9.0	O.D.
Wheat bran	-	0.06	14 mm	0.22	20 mm	0.03
Rice hulls	14	0.15	12 mm	0.08	12 mm	0.26
Soya bean meal	-	0.05	16 mm	0.17	-	0.05

Table.7 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soyabean meal at Varying pH (Incubation Period 72 hours)

Substrate	pH 4.0	O.D.	pH 7.0	O.D.	pH 9.0	O.D.
Wheat bran	-	0.04	-	0.36	18 mm	0.04
Rice hulls	12 mm	0.52	-	0.08	-	0.04
Soya bean meal	13 mm	0.23	13 mm	0.10	-	0.20

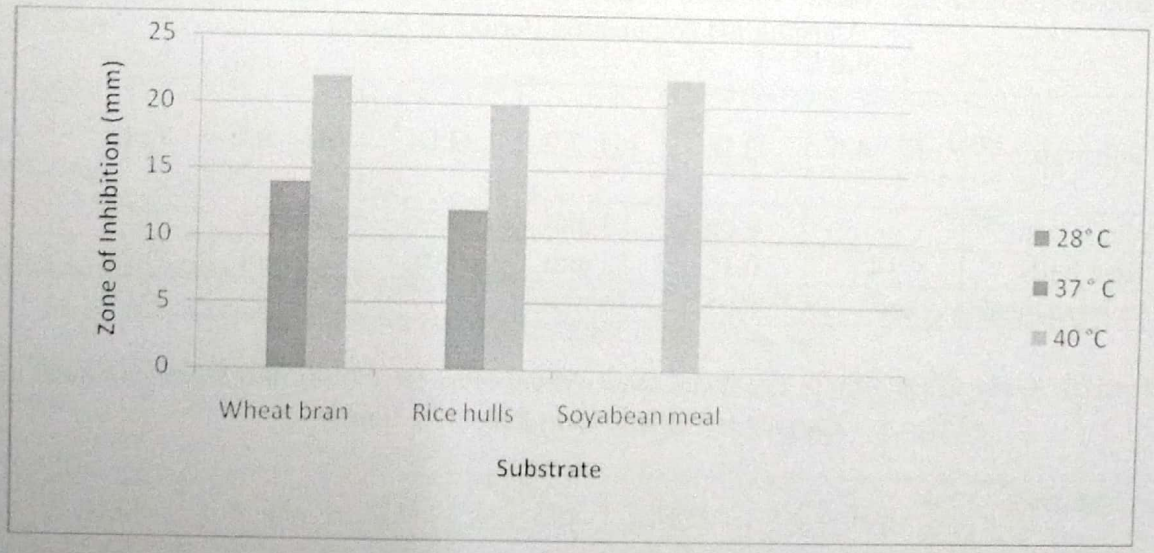
Table.8 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soyabean meal at Varying pH (Incubation Period 96 hours)

Substrate	pH 4.0	O.D.	pH 7.0	O.D.	pH 9.0	O.D.
Wheat bran	16 mm	0.08	-	0.06	-	0.12
Rice hulls	-	0.12	12 mm	0.06	-	0.03
Soya bean meal	12 mm	0.06	13 mm	0.15	-	0.04

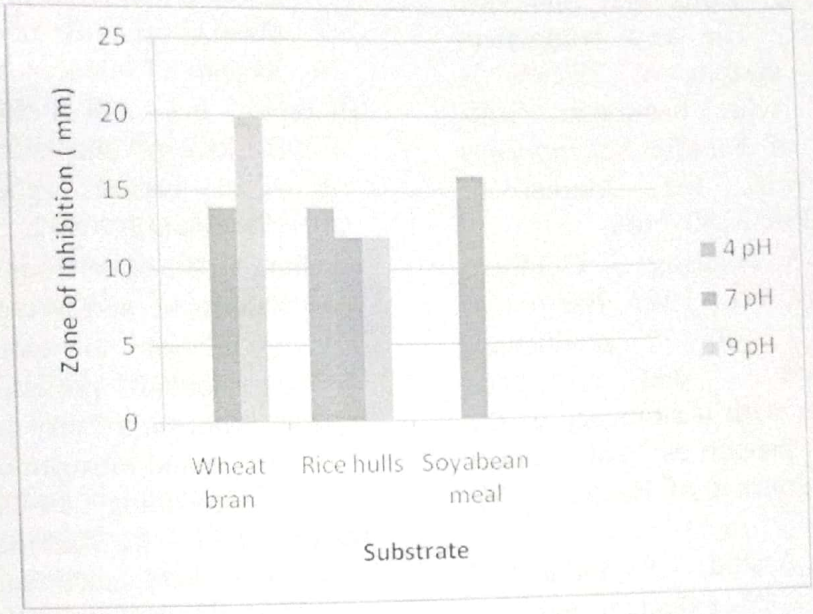
Table.9 Assay of Bacitracin Produced from Wheat bran, Rice hulls and Soyabean meal at Varying Time (at 37^0C, pH 7)

Substrate	24 hrs	O.D.	48 hrs	O.D.	72 hrs	O.D.	96 hrs	O.D.
Wheat bran	20 mm	0.38	14 mm	0.22	-	0.36	-	0.06
Rice hulls	-	0.09	12 mm	0.08	-	0.01	12 mm	0.06
Soya bean meal	19 mm	0.16	-	0.17	13 mm	0.10	13 mm	0.15

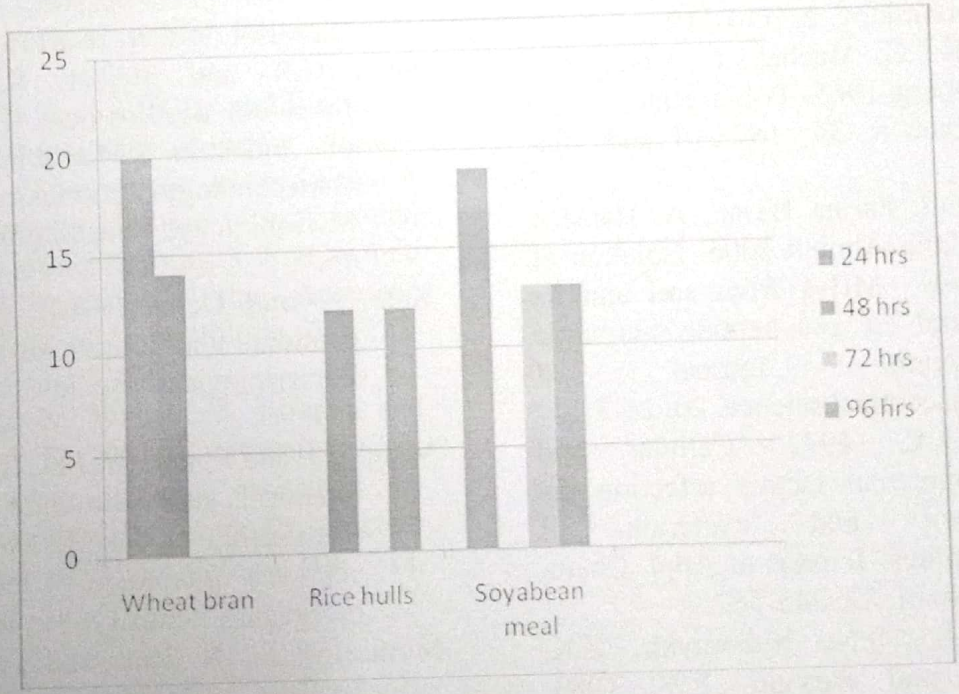
Graph.1 Assay of Bacitracin Produced by *Bacillus licheniformis* against *Staphylococcus aureus* at Varying Temperature (Incubation Period 48 hours)



Graph.2 Assay of Bacitracin Produced by *Bacillus licheniformis* against *Staphylococcus aureus* at Varying pH (Incubation Period 48 hours)



Graph.3 Assay of Bacitracin Produced by *Bacillus licheniformis* against *Staphylococcus aureus* at Varying Time (At 37^0C, pH 7.0)



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