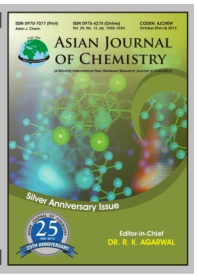
Asian Journal of Chemistry; Vol. 25, No. 18 (2013), 10493-10498

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2013.15761



Optimization and Enhanced Production of Hygromycin B Under Solid State Fermentation from *Streptomyces hygroscopicus* MTCC 1105

NARAYANAN MAHESH*, SRINIVASAN BALAKUMAR, MANI ARUNKUMAR, GANESAN SEETHA and PADMANABAN CHITRA

Department of Chemistry and Biosciences, Srinivasa Ramanujan Centre, SASTRA University, Kumbakonam-612 001, India

*Corresponding author: Fax: +91 435 2402460; Tel: +91 435 2426823; E-mail: magi.mbbt@src.sastra.edu

(*Received*: 13 May 2013; *Accepted*: 22 November 2013) AJC-14434

In the current study, four substrates such as wheat rawa, bombay rawa, rice bran and barley were screened for the ability to produced hygromycin B under solid state fermentation. The substrates, bombay rawa produce the highest yield when compared to other substrate in solid state fermentation. The present research showed the maximum yield was obtained in bombay rawa as 966 µg/g. Similarly the moisture 60 % showed 495 µg/g, pH 7 as 451.2 µg/g, temperature 28 ºC produces 495 µg/g, incubation period 6 days of 552.6 µg/g, 1 % w/w soluble dextrose of 930 µg/g, yeast extract of 585 µg/g and ammonium sulphate of 586.8 µg/g were obtained and optimized. Antibiotic sensitivity test assay proved that both gram positive and negative microorganisms are sensitive to hygromycin B. Among the substrates, bombay rawa showed the maximum zone of inhibition in (35 mm) diameter against *Klebsiella pneumoniae*. These results showed the way to the pharmaceutical industries for the development of products under solid state fermentation.

Key Words: Hygromycin B, *Streptomyces hygroscopicus* MTCC 1105.

INTRODUCTION

Most of the strains which produces antibiotics are generally exhibiting the resistance to the antibiotics which they produced[1]. They bind to specific sites on the ribosome and affect the ribosomal translation cycle. The modes of action of some antibiotics are now well recognized[2,3]. The development of resistance through the mechanism of modifying the target region like erythromycin, thiostrpton and also self modification of antibiotics by enzymatic has been recorded for a number of aminocyclitols[2,4-6]. The microbial enzymes are responsible for aminoglycoside antibiotic resistance by inactivation through phosphorylation, adenylation or acetylation[7-9].

Our previous studies reported that *Streptomyces* is capable of suppressing the growth of Gram-negative and Gram-positive bacteria widely[10]. Hence, the present study orients towards the utilization of *Streptomyces* for the production of hygromycin B.

Hygromycin B is one of the aminoglycoside antibiotics which are produced by the *Streptomyces hygroscopicus.* It has the dynamic effect on both prokaryotic and eukaryotic cells by affecting the polypeptide synthesis. It stabilizes the tRNA ribosomal acceptor site and stops the translocation process. The aminocyclitols, N-methyl-2 deoxy streptamine, is linked by a β-glycosidic bond to the talose sugar. The final moiety is bound by *ortho*-ester formation between the group and destomic acid. However, the obvious mechanism of hygromycin B to arrest the protein synthesis by the ribosome is not known even though with the years of passionate study.

When compare to submerged fermentation, the solid state fermentation is yielding higher amount of secondary metabolites. The solid state fermentation process is free of water and near to the natural environment to which microbes are adopted. The solid state fermentation has been used for the enzyme and secondary metabolites production[11,12].

Various studies of solid state fermentation factors like pH, temperature, inoculum size, incubation time, carbon sources and nitrogen sources were analyzed. Four different substrates banana peel, garlic peel, wheat bran and rice bran were used. The earlier study revealed that a modified solid-state fermentation was used to produce mevastatin by *Penicillium citrinum* NCIM 768 using wheat bran as the carrier[13-15]. Hence, this paper describes the production of hygromycin B and its partial HPLC analysis of culture filtrate and partial characterization and optimization of the bioactive compounds.

EXPERIMENTAL

Microorganism: *Streptomyces hygroscopicus* MTCC 1105 were acquired from the Institute of Microbial Technology (IMTECH) Chandigarh, India and maintained on ISP2 agar slant. Sub culturing was done by the subsequent intervals.

Substrate for antibiotic production: Commercial quality of wheat rawa, bombay rawa, barley and rice bran were purchased

from a local market. Each 10 g of solid substrates were used for the production of the hygromycin B through solid state fermentation.

Salt solution: In addition to nutrient, 1 mL of the salt solution (K_2HPO_4 - 0.5 g/L, $MgSO_4 \cdot 7H_2O$ - 0.5 g/L, $FeSO_4 \cdot 7H_2O$ - 0.5 g/L, NaCl-0.5 g/L) was added to the each substrate.

Optimization parameters under solid state fermentation: By varying any of the parameters like initial moisture, incubation time/temperature, pH and additives of varying carbon and nitrogen source at a time are influencing factors for optimization process for hygromycin B production.

Estimation of moisture content: Drying 10 g of solid substrate to constant weight at 80 ºC and dry weight was analyzed. Fixing of the initial moisture content is performed by soaking the substrate with known quantity of water and drying it again and calculated.

Moisture content (initial) of solid medium = (wt. of the substrate- dry wt.) × 100/ dry wt.

Effect of moisture content, incubation temperature, period and pH: Initial moisture content adjusted to 50, 60, 70 and 80 %, respectively with the addition of 5 % inoculum for fermentation at 28 ºC is to be monitored for 4 days. Simillarly, the fermentation process to be studied at 25, 28, 37 and 50 ºC.

With the incubation period of 4, 6, 8 and 10 days the fermentation carried out at pH 7 and 28 ºC were maintained. Varying the pH 5-8 with 1 N HCl or 1 N NaOH and the process monitored at 28ºC is to be monitored for 4 days to study the effect of pH.

Effect of carbon source and supplementary nitrogen source: Keeping all other conditions at optimum level the effect of hygromycin B is checked with carbon sources like maltose, fructose, starch, dextrose and lactose. Equally the study is carried out and the yield studied by using different supplementary nitrogen sources such as tryptone, peptone, yeast extract and casein. Additionally inorganic nitrogen sources such as $NaNO_3$, $(NH_4)_2SO_4$, KNO_3 and K_2HPO_4 were also studied.

Analytical methods for solid state fermentation

Antibiotic extraction and sensitivity study: The supernatant liquid obtained at centrifuging the substrate at 6000 rpm for 0.5 h and equal volume of filtrates was extracted with ethyl acetate using separating funnel. The crude extract is obtained and tested for its antimicrobial activity against specific pathogens such as *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Bacillus subtilis* and *Pseudomonas aeruginosa* by swabbed in sterile petri dishes containing the Muller Hinton agar medium and incubated for 24-48 h for measuring the incubation zones.

Absorption maximum on hygromycin B: The absorption maximum of (λ_{max}) the hygromycin B was identified as 210 nm by variable scanning mode using UV-visible spectrophotometer. Similarly the samples were analyzed by 210 nm and it was quantify with respect to standard graph plot with different concentration of standard.

Purification and quantification of antibiotic from HPLC: Antibiotic study was carried out using a Shimadzu liquid chromatography with at 254 nm. Antibiotics were eluted with a Shimadzu C_{18} reverse phase column (30 cm by 4 mm) with relevant pre column to predict the integrity of the compound. A suitable organic aqueous mobile phase at flow rate of 1.5 mL/min used.

RESULTS AND DISCUSSION

Solid state fermentation: The solid substrate assumes its importance in the screening and renewable source of agricultural waste for the growth of microbes as well as for the formation of industrially important products.

Optimization parameters under solid state fermentation

Effect of moisture content, incubation temperature, period and pH: The present research concludes 60 % moisture gave the maximum yield of 495 µg/g on bombay rawa followed by wheat rawa 363 µg/g, rice bran 433.8 µg/g, barley 392.4 µg/g (Fig. 1a). At 28 ºC, the maximum yield of hygromycin B was achieved in bombay rawa (495 µg/g) followed by wheat rawa (451.2 µg/g), rice bran (405 µg/g) and barley (432.6 µg/g) (Fig. 1b).

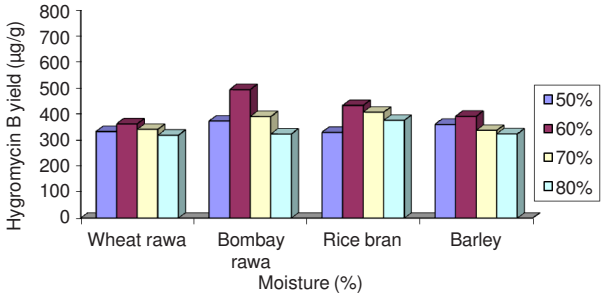


Fig. 1. (a) Effect of moisture content on hygromycin B production

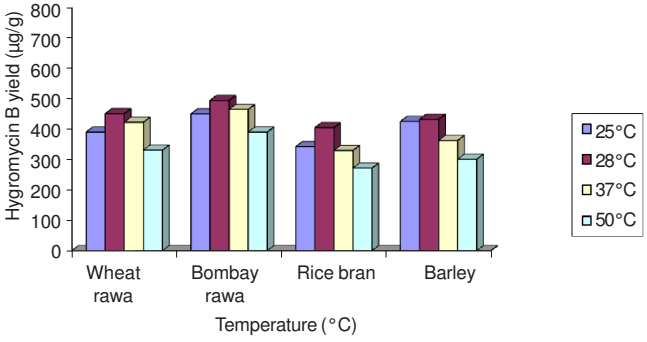


Fig. 1. (b) Effect of incubation temperature on hygromycin B production

Fig. 1c explained that various incubation period showed the significant effect of hygromycin B. Among the different incubation period, 6 days of incubation gave maximum amount of hygromycin B (552.6 µg/g) on bombay rawa and followed by wheat rawa (510 µg/g), rice bran (486 µg/g) and barley (450 µg/g). The effect of pH is an important factor for production of Hygromycin B. Present research concludes pH 7 showed the maximum yield of hygromycin B (451.2 µg/g) was achieved by *S. hygroscopicus* on bombay rawa followed by wheat rawa (420.6 µg/g), rice bran (409.8 µg/g) and barley (403.2 µg/g) (Fig. 1d).

Effect of carbon source and supplementary nitrogen source: Sugars like dextrose, maltose, fructose, lactose and starch at 1 % w/w as additives resulted higher in bombay rawa

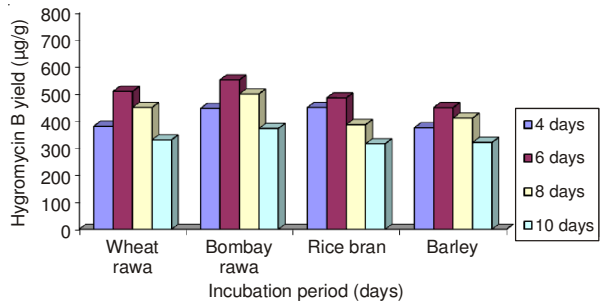


Fig. 1. (c) Effect of incubation period on hygromycin B production

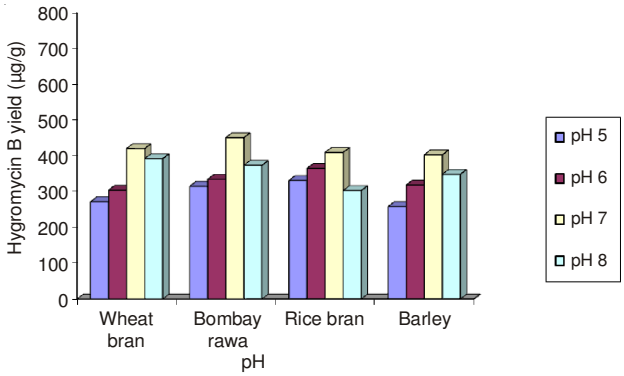


Fig. 1. (d) Effect of pH on hygromycin B production

and 930, 769, 822, 916.8 and 606 µg/g for the respective sugars (Fig. 1e). The maximum hygromycin B yield (585 µg/g) was obtained on bombay rawa with yeast extract as the nitrogen additives, whereas, wheat rawa (519 µg/g), rice bran (480.6 µg/g) and barley (435 µg/g) (Fig. 1f). The maximum antibiotic yield (586.8 µ/g) was obtained on bombay rawa with ammonium sulphate followed by potassium nitrate (516 µg/g), sodium nitrate (534 µg/g) and diammonium hydrogen phosphate (450 µg/g) for different inorganic phosphates (Fig. 1g).

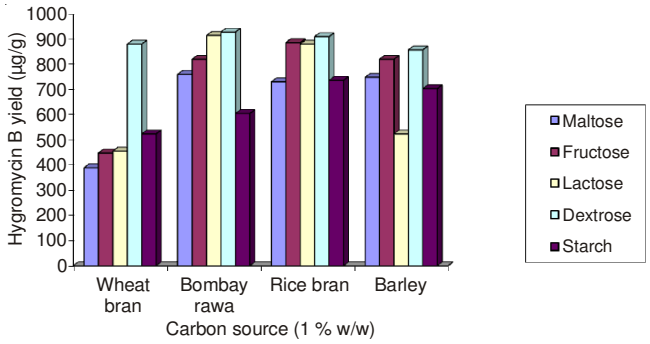


Fig. 1. (e) Effect of carbon source on hygromycin B production

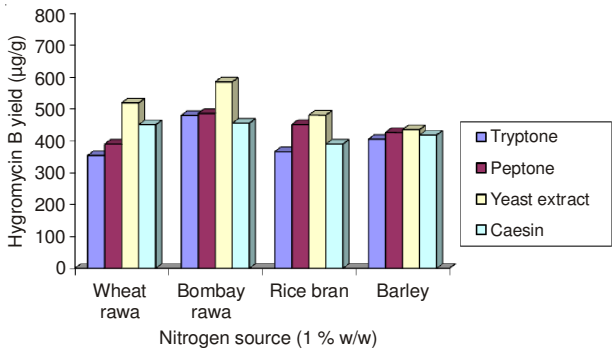


Fig. 1. (f) Effect of supplementary nitrogen source on hygromycin B production

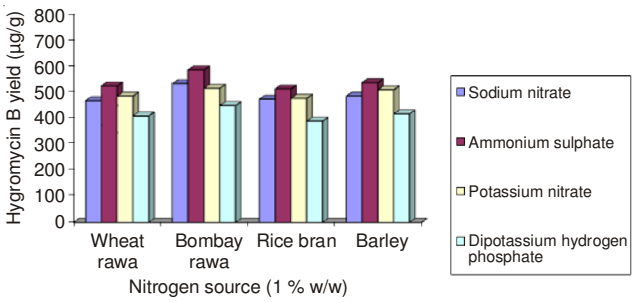


Fig. 1. (g) Effect of inorganic nitrogen source on hygromycin B production

Antibiotic sensitivity test assay on different parameters

Moisture: The antibiotic sensitivity test proved both Gram positive and Gram negative microorganism are sensitive to hygromycin B at 60 % of moisture content (Table-1), showed that maximum zone of inhibition (26 mm) in diameter against *K. pneumoniae* and *P. aeruginosa* followed by *S. aureus* (19 mm) , *E. coli* (19 mm) and *B. subtilis* (24 mm).

The maximum zone of inhibition (24 mm) in diameter against the *S. aureus* at 60 % of moisture level[16].

Incubation temperature: The effect of temperature was studied at 25, 28, 37 and 50 ºC. The optimal temperature at 28 ºC showed the maximum zone of inhibition (24 mm) in diameter against *K. pneumoniae* followed by *S. aureus* (14 mm), *E. coli* (16 mm), *P. aeruginosa* (17 mm), *B. subtilis* (12 mm) in the substrate bombay rawa (Table-2).

The maximum zone of inhibition (18 mm) in diameter against the *S. aureus* at 30 ºC[17].

Incubation periods: The antibiotic produced at among the different substrates, bombay rawa showed the maximum zone of inhibition in (20 mm) diameter against *K. pneumoniae and P. aeruginosa* followed by *S. aureus* and *E. coli* (14 mm), *B. subtilis* (17 mm) (Table-3).

The maximum zone of inhibition (24 mm) in diameter against the *B. subtilis* was found on 10th day of incubation[18].

pH: The effect of pH for antibiotic sensitivity test assay proved that pH 7 showed the maximum zone of inhibition (26 mm) in diameter against *K. pneumoniae* followed by *S. aureus* (20 mm), *E. coli* (17 mm), *P. aeruginosa* (24 mm) and *B. subtilis* (19 mm) in the substrate bombay rawa (Table-4).

The effect of pH and antimicrobial metabolite production by the strain *P. aeruginosa,* the optimum pH was 7. The maximum zone of inhibition was 23 mm[19].

Carbon sources: Among all the various substrates, bombay rawa inoculated with dextrose as additive medium showed that maximum zone of (22 mm) in diameter against *K. pneumoniae* followed *by S. aureus* (18 mm), *E. coli* (13 mm), *P. aeruginosa* (19 mm) and *B. subtilis* (14 mm) (Table-5).

The impact of different carbon sources and antibiotic production by the strain against *P. aeruginosa*, the carbon sources was maltose. The maximum zone of inhibition was 14 mm[19].

Supplementary nitrogen source: The maximum zone of inhibition (27 mm) showed on bombay rawa with yeast extract as supplementary nitrogen additives against *K. pneumoniae* (27 mm), *S. aureus* (16 mm), *E .coli* (17 mm), *P. aeruginosa* (24 mm) and *B. subtilis* (20 mm) (Table-6).

TABLE-1
ABST ASSAY FOR DIFFERENT MOISTURE CONTENT IN SSF

S. No.	Pathogenic microorganisms	Zone of inhibition (mm)															
		50 % moisture content				60 % moisture content				70 % moisture content				80 % moisture content			
		BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B
1	*Staphylococcus aureus*	16	10	11	14	19	14	17	16	15	13	11	12	17	14	17	15
2	*Escherichia coli*	17	14	12	14	19	15	17	16	16	12	14	12	19	15	15	12
3	*Pseudomonas aeruginosa*	25	10	12	10	26	15	24	21	22	14	10	18	21	15	20	14
4	*Bacillus subtilis*	20	13	10	11	24	19	19	13	19	14	12	11	17	10	11	16
5	*Klebsiella pneumoniae*	30	28	17	18	26	22	21	20	35	17	27	15	24	20	20	13
6	Standard (Hygromycin B)	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

*BR-Bombay rawa; WR-Wheat rawa; RB-Rice bran; B-Barley.

TABLE-2
ABST ASSAY FOR DIFFERENT TEMPERATURE

S. No.	Pathogenic microorganisms	Zone of inhibition (mm)															
		25 ºC				28 ºC				37 ºC				50 ºC			
		BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B
1	*Staphylococcus aureus*	19	15	17	18	14	10	12	11	20	18	16	15	17	15	14	16
2	*Escherichiacoli*	14	10	13	10	16	14	15	13	14	10	10	11	12	11	10	12
3	*Pseudomonas aeruginosa*	16	14	15	12	17	15	16	10	12	10	11	10	20	14	11	10
4	*Bacillus subtilis*	17	17	12	10	12	10	10	13	16	10	10	10	24	14	19	17
5	*Klebsiella pneumoniae*	20	17	19	16	24	17	22	15	27	16	15	16	26	16	17	14
6	Standard (Hygromycin B)	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

*BR-Bombay rawa; WR-Wheat rawa; RB-Rice bran; B-Barley.

TABLE-3
ABST ASSAY FOR ISOLATED ANTIBIOTICS WITH DIFFERENT INCUBATION PERIOD

S. No.	Pathogenic microorganisms	Zone of inhibition (mm)															
		4^{th} day of incubation period				6^{th} day of incubation period				8^{th} day of incubation period				10^{th} day of incubation period			
		BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B
1	*Staphylococcus aureus*	12	10	10	12	14	11	13	11	19	18	19	10	18	18	17	18
2	*Escherichia coli*	12	10	11	10	14	13	13	12	12	11	11	11	13	11	12	10
3	*Pseudomonas aeruginosa*	16	10	12	13	20	18	20	17	16	14	13	12	19	13	17	12
4	*Bacillus subtilis*	15	12	15	13	17	12	13	12	13	10	10	11	14	12	10	13
5	*Klebsiella pneumoniae*	20	14	18	17	20	19	15	17	20	18	19	10	18	14	18	13
6	Standard (Hygromycin B)	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

*BR-Bombay rawa; WR-Wheat rawa; RB-Rice bran; B-Barley.

TABLE-4
ABST ASSAY FOR DIFFERENT pH

S. No.	Pathogenic microorganisms	Zone of inhibition (mm)															
		pH 5				pH 6				pH 7				pH 8			
		BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B
1	*Staphylococcus aureus*	18	12	13	13	15	13	15	13	20	17	15	13	15	12	11	10
2	*Escherichia coli*	15	13	13	12	14	12	12	12	17	13	17	14	13	12	13	12
3	*Pseudomonas aeruginosa*	20	17	18	10	20	17	17	10	24	22	23	21	20	14	19	13
4	*Bacillus subtilis*	15	13	15	13	16	14	14	13	19	17	17	14	16	15	15	13
5	*Klebsiella pneumonia*	25	21	18	17	25	23	18	15	26	20	24	21	24	22	19	20
6	Standard (Hygromycin B)	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

*BR-Bombay rawa; WR-Wheat rawa; RB-Rice bran; B-Barley.

TABLE-5
ABST ASSAY FOR DIFFERENT CARBON SOURCE

S. No.	Pathogenic microorganisms	Zone of inhibition (mm)																			
		Maltose				Fructose				Lactose				Dextrose				Starch			
		BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B
1	*Staphylococcus aureus*	19	19	18	17	18	16	18	15	16	10	12	12	18	14	18	18	14	10	11	10
2	*Escherichia coli*	14	12	13	11	14	10	14	12	12	12	11	12	13	10	13	11	12	11	12	11
3	*Pseudomonas aeruginosa*	18	17	17	15	19	15	11	10	19	17	14	12	22	16	19	15	19	19	15	12
4	*Bacillus subtilis*	13	10	12	11	15	10	13	12	12	11	11	12	14	14	10	14	13	12	13	11
5	*Klebsiella pneumoniae*	20	19	15	13	18	16	16	15	18	14	17	16	19	15	18	18	18	15	16	15
6	Standard (Hygromycin B)	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

*BR-Bombay rawa; WR-Wheat rawa; RB-Rice bran; B-Barley.

TABLE-6
ABST ASSAY FOR DIFFERENT SUPPLEMENTARY NITROGEN SOURCE

S. No.	Pathogenic microorganisms	Zone of inhibition (mm)															
		Yeast extract				Peptone				Tryptone				Casein			
		BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B
1	*Staphylococcus aureus*	16	14	15	13	13	11	10	10	14	12	11	11	17	11	12	11
2	*Escherichia coli*	17	14	15	16	17	13	14	12	16	13	13	13	15	13	13	12
3	*Pseudomonas aeruginosa*	24	22	23	20	20	18	16	15	20	19	19	17	20	12	18	15
4	*Bacillus subtilis*	20	17	16	12	17	15	15	10	17	16	15	17	18	14	14	11
5	*Klebsiella pneumoniae*	27	21	22	20	24	20	18	15	23	16	21	17	23	15	20	18
6	Standard (Hygromycin B)	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

*BR-Bombay rawa; WR-Wheat rawa; RB-Rice bran; B-Barley.

The effect of nitrogen sources on antimicrobial metabolites production by the strain against *P. aeruginosa*, the nitrogen source was yeast extract. The zone of inhibition was 21 mm[19].

Inorganic nitrogen sources: The ABST assay proved that bombay rawa added with ammonium sulphate medium gave maximum zone of inhibition (23 mm) in diameter against *K. pneumoniae* and *S. aureus, E. coli* (22 mm), *P. aeruginosa* and *B. subtilis* (21 mm) (Table-7).

The effect of inorganic nitrogen sources on antimicrobial metabolites production by the strain against the *P. aeruginosa*, the inorganic nitrogen source was sodium nitrate. The zone of inhibition was 10 mm[19].

Purification and quantification of hygromycin B by HPLC: Considering HPLC analysis as a litmus test for purifying and quantified at 0.5 mg/mL with the retention period of 6.12 min (Table-8 and Fig. 2a-b).

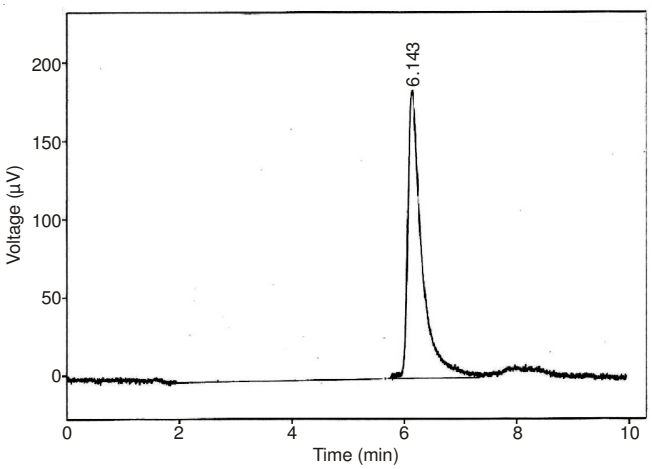


Fig. 2. (a) HPLC chromatogram of standard hygromycin B

Conclusion

The production of secondary metabolites are high value products of use in different pharamaceutical industries. In solid state fermentation the optimum productivity of hygromycin B (966 µg/g) was accomplished with bombay rawa through various process parameters optimized as indicated earlier.

Antibiotic sensitivity test assay proved that both Gram positive and Gram negative microorganisms are sensitive to hygromycin B. Among the substrates, bombay rawa showed the maximum zone of inhibition in (35 mm) diameter against *K. pneumoniae*. This study concludes solid state fermentation is the low cost and empirical technology for the pharmaceutical industries.

Moreover, hygromycin B production is possible through some other low cost substrate as wheat bran, sweet potato, rice and others. rDNA technology is to be used for the *S. hygroscopicus* to mutate the strain and can be used for higher hygromycin B through fermentor vessel. These substrates are also use in pharmaceutical industries to manufacture hygromycin B. Further, fermentors such as airlift and solid

TABLE-7
ABST ASSAY FOR DIFFERENT INORGANIC NITROGEN SOURCE

S. No.	Pathogenic microorganisms	Zone of inhibition (mm)															
		Sodium nitrate				Ammonium sulphate				Potassium nitrate				Diammonium hydrogen phosphate			
		BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B	BR	WR	RB	B
1	*Staphylococcus aureus*	20	19	17	18	23	21	20	18	23	20	18	17	23	18	20	17
2	*Escherichia coli*	21	19	19	20	23	20	20	18	20	18	17	17	21	19	20	17
3	*Pseudomonas aeruginosa*	21	18	17	20	21	18	20	17	20	18	17	16	23	20	21	19
4	*Bacillus subtilis*	21	20	18	20	21	18	17	15	20	17	18	16	23	19	20	18
5	*Klebsiella pneumoniae*	23	20	18	22	22	19	17	17	25	18	17	15	24	20	20	17
6	Standard (Hygromycin B)	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16

*BR-Bombay rawa; WR-Wheat rawa; RB-Rice bran; B-Barley.

TABLE-8
HPLC ANALYSIS FOR PURIFICATION AND QUANTIFICATION

Detector A (254 nm)					
PK #	Retention time (min)	Area	Height	Name of the compound	Concentration (µg/µL)
1	6.14	33.2	36.6	Standard hygromycin B	10.00
2	6.12	66.4	74.7	Hygromycin B	500

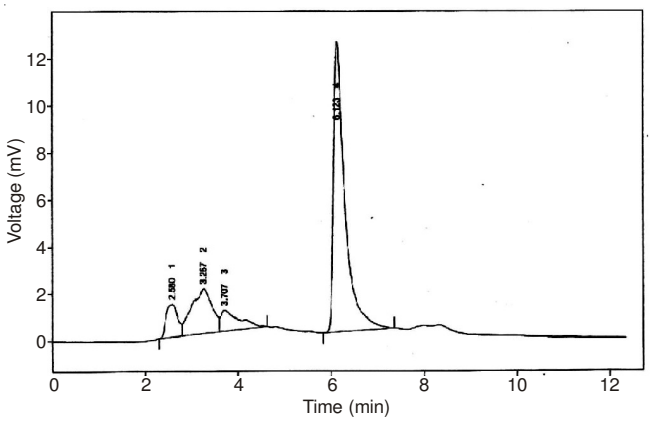


Fig. 2. (b) HPLC chromatogram of the sample-antibiotic from microbes

state fermentor may also be used to increase the production through optimization.

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