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OPTIMIZATION OF MEDIA FOR PRODUCTION OF BIOACTIVE COMPOUNDS BY *STREPTOMYCES PARVULLUS* SS23/2 ISOLATED FROM MARINE ALGAE IN THE BAY OF BENGAL, INDIA

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ABSTRACT: *Streptomyce*s are economically and biotechnologically valuable prokaryotes responsible for production of bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes. The present study deals with the optimization of media for production of bioactive compounds from *Streptomyces parvullus* SS23/2 isolated from marine algae (*Dictyota dichotoma*) at the Bay of Bengal, India. Suitable medium was selected and optimized under different chemical and physical parameters for maximum production following one-factor- at- a time approach. Optimum condition for initial pH, medium capacity, inoculum size, inoculum age, agitation and growth period were assessed and the result was found to be optimum at 7, 50 ml, 7.5% (v/v), 48 hours, 180 rpm and 6 days, respectively. Component of the best carbon source for the basal medium was sucrose at 2% and 1.5% concentration for bacteria and fungi, respectively. Malt extract at 1% concentration was the best nitrogen source for both bacteria and fungi. The composition of the best medium for *Streptomyces parvullus* SS23/2 was (g/l: Corn steep liquor, 10, sucrose, 20; malt extract, 10; K_2HPO_4, 5; NaCl, 2.5; $ZnSO_4$, 0.04; $CaCO_3$, 0.4; pH=7). After optimization, antimicrobial activity was improved from 36 mm to 40.7 mm inhibition zone against *B. subtilis*. In the research for bioactive compounds, screening, characterization and optimization of the media and cultural conditions are important for strain improvement. Therefore, optimization of products from *Streptomyces* in different samples may create a great potential for upscaling secondary metabolites such as antibiotics and might remain an area of research interest in the future antibiotic discovery.

Key words/phrases: *B. subtilis*, Marine sediment, Optimization, *Streptomyces parvullus*.

INTRODUCTION

Secondary metabolites are commonly present in organisms that lack an immune system and thus are rarely produced by higher animals (Maplestone *et al*., 1992). These compounds are, therefore, mainly produced by bacteria,

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fungi, algae, corals, sponges, plants and lower animals. Filamentous microorganisms are the main sources of secondary metabolites with nearly 75% of all described antibiotics being produced by actinomycetes and 17% by moulds.

Microbial natural products still appear as the most promising source of the future antibiotics, which are desperately needed. The arguments supporting this idea are the unparalleled structural diversity that can be found in nature, the fact that the natural antibiotics have apparently been shaped by evolution to make them effective in killing microorganisms, and the suggestions provide that the field is still much unexplored, in terms of microbial diversity, potential to trigger the expression of silent pathways by manipulating the cultivation conditions, and a number of molecular targets still to be exploited for antibiotic therapy (Wright and Sutherland, 2007; Pelaez, 2006). According to Berdy (1995), around 11,900 antibiotics had been discovered by 1994 of which around 6,600 (55%) were produced by *Streptomyces* species whereas filamentous fungi produced 2,600 (22%), bacteria produced 1,400 (12%) and non-*Streptomyces* strains of actinomycetes produced 1,300 (11%).

Among actinomycetes, *Streptomyces* populations are reported to be the most abundant group in soil. They produce potentially useful compounds providing the widest range and most promising array of pharmacologically and agriculturally active compounds. *Streptomyces* are widely recognized as industrially important microorganisms because of their ability to produce a variety of novel secondary metabolites including antibiotics (Bibb, 2005). They have provided more than half of the naturally occurring antibiotics discovered to date and continue to be screened for useful compounds (Miyadoh, 1993). It is clear that the effect of medium components (carbon and nitrogen sources) and physical factors such as incubation time, temperature and pH, etc have profound effect on secondary metabolite production (Himabindu and Jetty, 2006). Low concentration medium may lead to poor growth and poor yield and high concentration may lead to catabolite repression and toxic side effects. Therefore, an attempt was made to optimize the nutrient level and other physical factors using one-factor-at-a-time approach (Panda *et al.*, 2007) for optimum production of antibiotics by *Streptomyces parvullus* SS23/2 isolated from marine algae.

MATERIALS AND METHODS

Identification of *Streptomyces parvullus* SS23/2

Streptomyces parvullus SS23/2 was isolated from marine algae (*Dictyota dichotoma*) in the Bay of Bengal, India and selected as a potent isolate for optimization. The study period for optimization was from December 2009-February 2010. In brief, the identification of *Streptomyces parvullus* SS23/2 was as follows: the morphological, cultural, physiological and biochemical characterization of the isolate was carried out as described in International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). The morphological properties of the isolate were examined by using light microscope as well as scanning electron microscope. The cell wall fraction and sugar composition were analyzed as per the procedures described earlier (Lechevalier and Lechevalier, 1980; Hasegawa *et al*., 1983). The whole-cell sugar composition was determined as reported by Becker *et al*. (1965) and Lechevalier and Lechevalier (1980). All the test results were compared using standard identification key for classification (Buchanan and Gibbons, 1974; Williams *et al*., 1983; Langham *et al*., 1989; Williams *et al*., 1989).

Determination of antimicrobial spectrum

Cross-streak (Waksman *et al*., 1948) and agar-overlay (Williams *et al*., 1983) methods were used for primary screening for isolating organisms having antimicrobial activities.

Antimicrobial activities were assessed using nutrient agar for bacteria and potato dextrose agar for fungi by first inoculating the isolate at the centre of plate and incubating at 28°C for 5 days. Twenty four hours fresh sub-cultured test organisms of bacteria and 48 hours of fungal cultures were prepared. Then test organisms were streaked perpendicular to the *S. parvullus* SS23/2 isolate. Agar-overlay method was performed by spot inoculation of *S. parvullus* SS23/2 and incubated for 5 days. Spot inoculated 5 days-old colonies of *S. parvullus* SS23/2 were killed by inverting the plates over 1.5 ml of chloroform for 40 minutes. The dead colonies were overlaid with 5 ml of semi-solid nutrient agar (bacteria) and 5 ml of semi-solid potato dextrose agar (fungi) that has been inoculated with the test organism. After incubation, zone of inhibition around the colonies was recorded. *Streptomyces parvullus* SS23/2 was further subjected to submerged fermentation using inoculum (seed) medium and production medium. The antimicrobial activity of fermented products was assayed by agar diffusion technique (Grove and Randall, 1995). Antimicrobial activity was assessed using standard strains of *Staphylococcus aureus* (NCIM 2079),

Bacillus subtilis (NCIM 2063) and *Penicillium chrysogenum* (NCIM 738). In all tests, plates were incubated at 28°C for 48 hours for fungi and at 37°C for 24 hours for bacteria.

Effect of inoculum size and incubation period for production of bioactive compounds

Shake flask fermentation was run in 250 ml flask containing 50 ml of inoculum medium-I (g/l: Soya bean meal, 10; Corn steep liquor, 10; Glucose, 10; $CaCO_3$, 5.0; pH=7) and fermentation was run for 48 hours. One percent, 2.5%, 5%, 7.5%, 10%, 12.5% of the inoculum medium was transferred to 500 ml flasks containing 100 ml of production medium-I (g/l: Soya bean meal, 10; Corn steep liquor, 10; Glucose, 10; $CaCO_3$, 5.0; pH= 7). All flasks were operated at room temperature in a rotary shaker at 180 rpm. At every 12 hours interval for 72 hours supernatant sample was taken and extracted using ethyl acetate and then extracted products were concentrated using rotary evaporator. Each extract was then assessed for its antimicrobial activity against standard strains of *S. aureus*, *B. subtilis* and *P. chrysogenum* employing agar diffusion technique (Grove and Randall, 1995).

Impact of pH on production of bioactive compounds by *Streptomyces parvullus* SS23/2

The effect of pH on antibiotic production was studied by inoculating the strains in production medium and adjusting the pH of the medium at pH 5, 6, 7, 8, and 9 and subjecting to submerged fermentation. Samples of fermented product were extracted and concentrated and then assessed for their activity after 5 days fermentation.

Effect of Carbon and Nitrogen sources on production of bioactive compounds by *Streptomyces parvullus* SS23/2

To determine the best carbon sources 1% of each of glucose, fructose, lactose, jowar starch, soluble starch, potato starch and xylose was separately added to the basal medium (g/l: $NaNO_3$, 4; K_2HPO_4, 5; NaCl, 2.5; $ZnSO_4$, 0.04; $CaCO_3$, 0.4; pH=7). To study the effect of nitrogen sources 1% of each of yeast extract, malt extract, meat extract, urea, tryptophane, casein, ammonium sulphate, glutamic acid, DL-methionine, soya bean meal and sodium nitrate was separately added to the basal medium (g/l: Glucose, 25; K_2HPO_4, 5; NaCl, 2.5; $ZnSO_4$, 0.04; $CaCO_3$, 0.4; pH=7).

Determination of dry weight from the mycelial pellet of *Streptomyces parvullus* SS23/2

Streptomyces parvullus SS23/2 was first inoculated in the inoculum medium-I and incubated for 48 hours. Then 7.5% of inoculum medium was transferred to production medium. After 24 hours of fermentation 20 ml of fermented broth was withdrawn each day for 8 consecutive days. Then the whole content of each batch was centrifuged at 4000 rpm for 15 minutes. The mycelial pellet was dried in hot oven at 60°C for 24 hours and then the dried cell weight was determined (Narayana and Vijayalakshmi, 2008).

RESULTS AND DISCUSSION

One of the most potent bioactive producing strains of *Streptomyces* was isolated from samples of marine algae (*Dictyota dichotoma*) in the Bay of Bengal, India and identified as *Streptomyces parvullus* SS23/2. As presented in Fig. 1, antifungal and antibacterial activity of the bioactive compounds produced by *S. parvullus* SS23/2 shows variation.

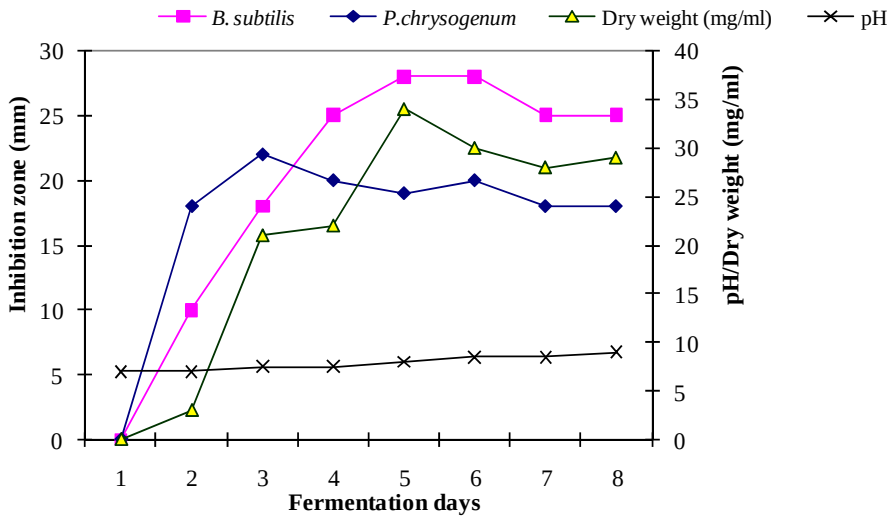

Fig. 1. Antimicrobial activity of *S. parvullus* SS23/2 against *B. subtilis* and *P. chrysogenum*.

That is, as dry weight increases the antibiotic production also increases up to the 5th day for *B. subtilis* and 3rd day for *P. chrysogenum*. As the dry weight declines, the antibacterial and antifungal activity also declines. As the pH increases, the effect of antifungal activity starts declining compared to antibacterial activity. The reason may be because as the length of fermentation time increases the pH of the medium increases (alkaline) and

the fungal cell may not be physiologically active due to high pH, which leads to a low interaction between the active compound and the fungal cells, resulting in less inhibition zone in *P. chrysogenum* than *B. subtilis*.

As presented in Figs. 2 and 3 among the different inoculum media tested, the appropriate medium used was IM-I which showed the better activity than IM-II for this particular isolate. Results on the effect of inoculum level and age of inoculum for optimum production of bioactive compounds by *S. parvullus* SS23/2 using 1%, 2.5%, 5.0% 7.5%, 10% and 12.5% concentration revealed that the appropriate inoculum's size for this isolate was found to be 7.5% followed by 10% and 5%. Maximum activity was recorded at age of 48 hours followed by 36 and 60 hours (Fig. 4).

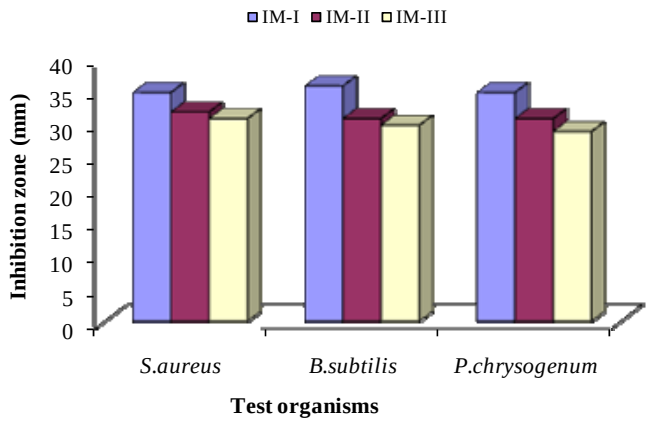

Fig. 2. Antibiotic production ability of *S. parvullus* SS23/2 in different inoculum media (IM) against *S. aureus*, *B. subtilis* and *P. chrysogenum.*

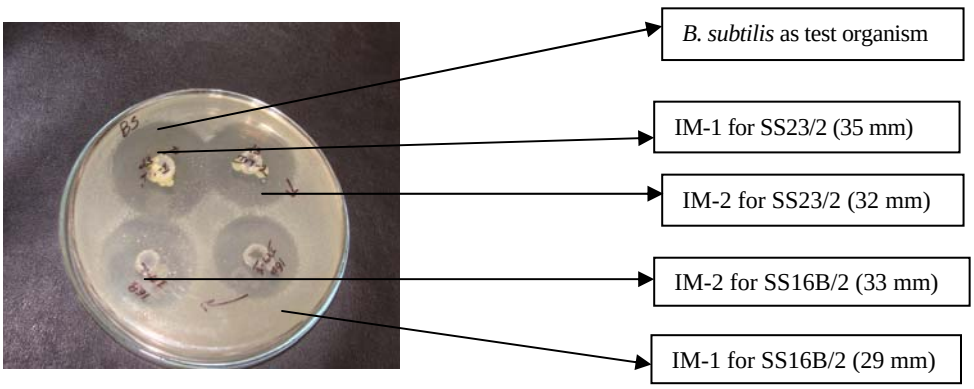

Fig. 3. Comparison of inoculum medium-I and II for antimicrobial activity between *S. parvullus* SS23/2 and other isolates of SS16B/2 against *B. subtilis*.

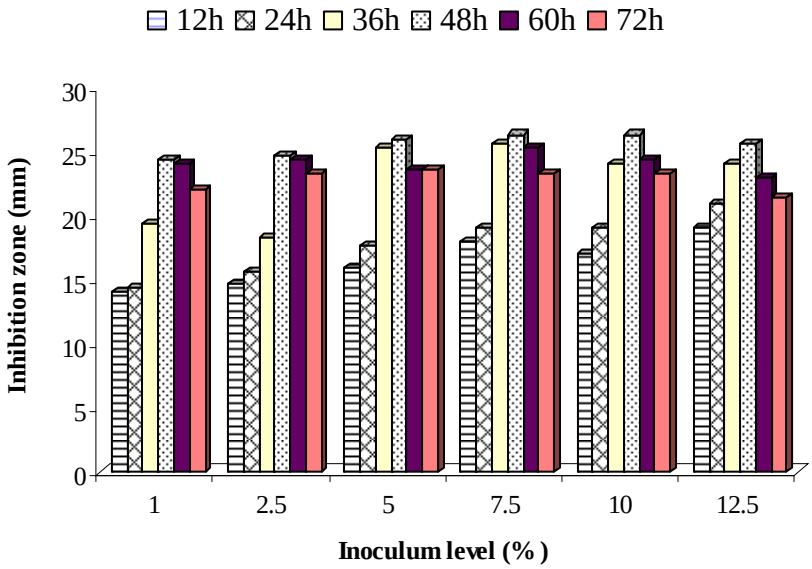

Fig. 4. Effect of inoculum size and age of inoculum for production of bioactive compounds by *S. parvullus* SS23/2 against *B. subtilis.*

Results on the effect of aeration and agitation for optimum production of bioactive compounds taking fermentation media with a working volume ratio (medium to flask ratio) and using different rpm revealed that 50 ml of fermentation broth at 180 rpm showed better production but this result was not significant. As described by Vieira *et al*., (2008) absence of agitation or aeration was determinant in the production of antibacterial metabolites. There was a higher cellular growth under agitation than for those which were kept static. The cell production was due to the homogenization and heat transfer when physical and chemical conditions are maintained homogenous and more oxygen becomes available (Mantzouridou *et al*., 2002).

The effects of carbon and nitrogen sources for antibiotic activity by *S. parvullus* SS23/2 were determined. Eight different carbon sources were studied by adding 1% concentration to the basal medium. There was a variation in the level of antimicrobial activity when the different carbon sources were tested. Glucose, fructose, sucrose, lactose, jowar starch, soluble starch, potato starch and xylose were carbon sources tested. Among all carbon sources tested, sucrose and lactose had a beneficial effect for better antibiotic production (Table 1).

Table 1. Role of carbon source on production of bioactive compounds by *S. parvullus* SS23/2 against *B. subtilis.*

Isolate	Carbon source (1%)	Biomass (mg/ml)	Inhibition zone (mm)				
			Flask-1	Flask-2	Flask-3	Mean	SD
SS23/2	Glucose (Positive control)	15.0	20	21	21	20.67	0.58
	Fructose	20.0	20	22	23	21.67	1.53
	Sucrose	**28.0**	**29**	**31**	**33**	**31**	**2.00**
	Lactose	23.0	29	30	31	30	1.00
	Jowar Starch	23.0	26	25	24	25	1.00
	Soluble starch	22.0	26	26	25	25.67	0.58
	Potato starch	23.0	25	23	21	23	2.00
	Xylose	18.0	27	26	25	26	1.00
	No carbon source (negative control)	16.0	18	17	16	17	1.00

It is very clear that the supplementation of different carbon, nitrogen, and amino acids altered the production of the intracellular and extracellular metabolites. In this study, it was observed that using sucrose as a carbon source increased the production of antibiotic for isolate *S. parvullus* SS23/2 followed by lactose, xylose, soluble starch and jowar starch. The minimum activity was obtained when using glucose and fructose. Our result is different from that of Demain and Fang (1995) who reported that carbohydrate such as glycerol, maltose, mannose sucrose and xylose interfered with the production of secondary metabolites. This may be due to species variation in which *Streptomyces* species can use different carbon source for their growth and production of secondary metabolites (Jonsbu *et al*., 2002).

It is well documented that glucose is usually an excellent carbon source for growth and interferes with the biosynthesis of many antibiotics such as bacitracin (Haavik, 1974) and actinomycin (Gallo and Katz, 1972). As reported by Martin and Demain (1980), during studies on fermentation medium development, polysaccharides or oligosaccharides were often found to be better than glucose as carbon sources for antibiotic production. As Gallo and Katz (1972) reported, a medium containing glucose plus a more slowly utilized carbon source, it was glucose that was usually used first in the absence of antibiotic production and when glucose was depleted, the second carbon source was used for antibiotic biosynthesis. Therefore, the reason why glucose and fructose showed low antibiotic activity in our study might be because these carbon sources are simple sugars that might be used initially (immediate energy source) for cell growth, not for later production of antibiotics or they may interfere with the biosynthesis of the antibiotic production that is produced by the isolate of *S. parvullus* SS23/2.

Eleven different nitrogen sources were studied by adding 1% concentration to the basal medium. It was observed that malt extract as nitrogen source increased the production of antibiotic for isolate SS23/2 followed by yeast extract, casein and sodium nitrate. The minimum activity was obtained in using urea, tryptophane and ammonium sulphate as nitrogen source (Table 2). As indicated by Marwick *et al*., (1999) some nitrogen sources like ammonium ion showed a strong inhibitory action on the production of both the antifungal and antibacterial metabolites. Among amino acids tested, tryptophane, methionine and glutamic acid were not good sources of nitrogen sources for our isolate. This may be because there are some compounds such as tryptone and glycerol which are stimulatory for antifungal metabolite production (Haque *et al*., 1995; Guerra and Pastrana, 2001) while other amino acids can inhibit or stimulate secondary metabolites of antibiotic production (Cheng *et al*., 1995).

Table 2. Role of nitrogen source on antibiotic production by *S. parvullus* (SS23/2) against *B. subtilis*.

Isolate	Nitrogen source (1%)	Inhibition zone (mm)				
		Flask-1	Flask-2	Flask-3	Mean	SD
SS23/2	Yeast extract	25	26	23	24.67	1.53
	Malt extract	**27**	**28**	**28**	**27.67**	**0.58**
	Meat extract	18	18	19	18.33	0.58
	Urea	15	18	14	15.67	2.08
	Tryptophane	15	15	17	15.67	1.15
	Casein	20	21	22	21.00	1.00
	Ammonium sulphate	16	15	16	15.67	0.58
	Glutamic acid	17	18	16	17.00	1.00
	DL-methionine	17	16	16	16.33	0.58
	Soya bean meal	18	17	16	17.00	1.00
	Sodium nitrate	21	21	20	20.67	0.58

During the study of antibiotic activity for isolate SS23/2 against *B. subtilis*, using different production media, maximum inhibition zone was recorded to be 36 mm. After optimization of the different physical and chemical parameters, the maximum inhibition zone observed was 40.7 mm for the same organisms. This showed that 0.13 fold or 13.0% improvement was observed in the production medium after optimization.

CONCLUSION

Component of the best carbon and nitrogen source for the basal medium was found to be sucrose and malt extract in a concentration of 2% and 1%, respectively, for bacteria. However, 1.5% sucrose and 1% malt extract showed better activity for fungi. The components of the best medium for our isolate was (g/l: Corn steep liquor, 10, sucrose, 20; malt extract, 10; K_2HPO_4, 5; NaCl, 2.5; $ZnSO_4$, 0.04; $CaCO_3$, 0.4; pH=7). In the research for bioactive compounds, screening, characterization and optimization of the

media and cultural conditions are important for strain improvement. Therefore, optimization of products from *Streptomyces* in different samples may create a great potential for upscaling secondary metabolites such as antibiotics and might remain an area of research interest in the future antibiotic discovery.

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