Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 7 (4): 227-234, 2012
ISSN 1816-4935 / DOI: 10.3923/jm.2012.227.234

Rifamycin SV Production Using Cereal Bran as Solid Substrate with *Amycolatopsis mediterranei*

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ABSTRACT

Application of rifamycin as a broad spectrum antibiotic, due to its demand and high consumption is increasing in human therapy. Continuous efforts are being made to produce it in a cheaper way using different available raw materials. In the present study, agroindustrial residual products like cereal bran were screened for the production of rifamycin SV with *Amycolatopsis mediterranei* by solid state fermentation. Optimization of various cultural and physiological parameters using cereal bran was carried out in conventional method by one parameter at a time method. The results indicated that at 90% initial moisture level and optimum cultural conditions, the production of rifamycin SV was higher with finger millet bran as substrate and showed a yield of 246 mg/100 gds while using glucose as a carbon source. This study confirms that cereal bran can perform as well solid substrate comprising of available nutrients along with a suitable carbon source for various antibiotic and metabolite fermentations thereby decreasing the production costs.

Key words: *Amycolatopsis mediterranei*, cereal bran, rifamycin SV, solid state fermentation

INTRODUCTION

The rifamycins (Oppolzer and Prelog, 1973; Jin *et al*., 2004) commercially in use rifampicin, rifabutin, rifapentine etc. are one of the most potent and broad spectrum antibiotics against bacterial pathogens mainly *Mycobacterium* sps and a key component of leprosy and AIDS related mycobacterial infections (Sepkowitz *et al*., 1995), anti-tubercular therapy in combination with Isoniazid and Streptomycin. They belong to ansamycin group of antibiotics (Rinehart and Shield, 1976) and exhibits antimicrobial and antiviral activity by inhibiting DNA dependant RNA polymerase and reverse transcriptase of certain RNA viruses respectively. Rifamycin SV is a key intermediate in the synthesis of rifampicin (Maggi *et al*., 1966) and other derivatives of therapeutic value (Sensi and Thiemann, 1967; Krishna *et al*., 1999).

The genus *Amycolatopsis* belonging to the family Pseudonocardiaceae comprises of about 120 bioactive microbial metabolites, exploited for ansamycin group of antibiotics mostly rifamycins (Raja and Prabakarana, 2011). Due to its importance in human therapy, its demand is tremendously increasing worldwide and continuous efforts are being made to decrease its

production cost by process optimization using cheaper raw materials through different fermentation processes like Submerged Fermentation (SmF) and Solid State Fermentation (SSF). Inexpensive culture media like left over crop residues, agroindustrial waste products have been found potential for biotechnological processes. Their usage has the potential to reduce cost of raw material and up to 60% product formation costs (Lotfy, 2007) during fermentations. Application of agro-based products in SSF has been found potential use in biotechnological processes (Pandey and Soccol, 1998). The importance of agroindustrial residues in SSF system for the production of enzymes and secondary metabolites has gained recognition in recent years (Holker *et al*., 2004; Singh *et al*., 2009). Different industrially useful metabolites are produced using SSF world wide. SSF is a technology so far is run only on a small scale, but has an advantage over SmF. In some cases, the production of antibiotics by SmF requires high energy and capital investment (Lal and Lal, 1994; Ghosh, 1992). Antibiotic production by SSF requires low energy, less capital investment and recently it has gained increased importance due to higher fermentation productivity and lesser disadvantages when compared with SmF.

Cereal and millet bran, by-products of milling technology are available plenty in India. They are ideal, cheap sources and mainly used as animal feed due to their high nutritive value and low cost. Millets are cultivated in a wide range of soils and climates and because of their short growing seasons, they are of specific importance in semi-arid regions including Andhra Pradesh. Finger millet (*Eleusine coracana*) locally known as ragi and by various other names worldwide are predominantly starchy like other cereals. The present study aims for the production of rifamycin SV by *Amycolatopsis mediterranei* NRRL ISP-5501 using agroindustrial residues like bran of few cereals with optimization of various cultural conditions by conventional method.

MATERIALS AND METHODS

Collection of substrates: Agroindustry residual substrates like wheat bran, corn husk, black gram bran, green gram bran, rice bran, *Phaseolus* gram bran, finger millet bran, pigeon pea bran etc. were procured from the local market, Hyderabad, India. Other chemicals and media ingredients were procured from Himedia and Qualigens, India.

Organisms used: *Amycolatopsis mediterranei* NRRL ISP-5501 and *Bacillus subtilis* NRRL B-354, kindly provided by United States Department of Agriculture, ARS culture collection (NRRL) centre, Peoria.

Growth medium: Inoculum was prepared in growth media (g L^{-1}) (Ghisalba *et al*., 1994) and the pH was adjusted to 7.0 and autoclaved at 121°C for 15 min.

Inoculum preparation: Inoculum was prepared by inoculating growth medium with four days old slant culture of *A. mediterranei*. A 5 mL of growth medium was transferred into 250 mL Erlenmeyer flask containing 100 mL of sterile inoculum medium. The flask was incubated on a rotary shaker at 200 rpm at 28°C for 5 days and used as an inoculum for further studies.

Media preparation for substrate fermentation: A 10 g of dry substrate was taken in 250 mL conical flasks and the initial moisture content was calculated for solid substrate using the formula:

$$\text{Moisture content (\%)} = \frac{(W1 - W2)}{W1} \times 100$$

The initial moisture content of the substrate was adjusted and checked at different moisture levels initially and 90% moisture level was maintained for further experiments using the wetting agent. The pH was set at 7.0 and autoclaved for 30 min at 121°C. After cooling the flasks to room temperature, preliminarily 30% inoculum was added and incubated at 28°C for nine days under static conditions. Similar method was carried out for each parameter to be optimized.

Extraction of rifamycin SV: After fermentation, the solid mass was soaked in 100 mL sterile 0.1 M phosphate buffer (pH 8.0) and agitated thoroughly on a shaker at 120 rpm for 30 min at 30°C and was stored at 4°C for 6 h. The resulting extract was centrifuged at 3000 rpm for 15 min and the clear supernatant was used for the estimation of rifamycin SV by Spectrophotometry and HPLC. The culture broth was subjected to bioassay using *Bacillus subtilis* as a test organism for its antibacterial activity.

Analysis of rifamycin SV by spectrophotometry: Rifamycin SV in the fermentation extracted solution was determined (Pasqualucci *et al*., 1970). A 1 mL fermentation solution was diluted in 5 mL of acetate buffer at pH 4.63 and the blank incorporated with 0.1% sodium nitrite in the same buffer to be read at 447 nm using UV-Vis spectrophotometer, make: Spectronics, Japan.

High performance liquid chromatography (HPLC): The fermentation broths were analyzed for rifamycins according to the method described by Vohra and Dube (1989). Samples were eluted with solution containing potassium phosphate buffer and acetonitrile. The column effluents were monitored using reverse phase column RP-C18 at 254 nm with retention time of 4.7 min at column temperature of 25°C.

RESULTS AND DISCUSSION

Evaluation of various solid substrates and effect of physiological and nutritional parameters for rifamycin SV production: Various cereals bran was used as substrate in the present study by autoclaving for thirty minutes at 121°C. In earlier reports, wheat bran was the best supported solid substrate for rifamycin SV production of approximately 300% increase after pretreatment or acid hydrolysis of the substrate using hydrochloric acid (Krishna *et al*., 1999). Similar reports of maximum production of rifamycin B were reported using wheat bran as a sole substrate (Venkateswarlu *et al*., 1999) and for cyclosporin production without pretreatment (Nisha and Ramasamy, 2008) mentioning the ability of the water retaining capacity of wheat bran among various substrates. In the present study, other substrates like green gram bran and rice bran showed less production of rifamycin SV which may be due to faster depletion and utilization of the sugars released from the solid support by the organism during fermentation. Finger millet bran showed a maximum yield of rifamycin SV (Fig. 1) (230 mg/100 gds) followed by wheat bran (218 mg/100 gds) and corn husk (186 mg/100 gds) which were reported as best substrate for rifamycin B. Similarly, report on barley bran use as a potential substrate for metabolite production was mentioned in comparison to other substrates by Soliman *et al*. (2012). Unlike other cereals, finger millets are predominantly starchy with huge contents of minerals, high calcium content and other nutrients. Even the bran which contains a pool of B-complex vitamins must have been favored the growth and production of rifamycin SV in fermentation studies.

Moisture content of the substrate is the most important factor in solid state fermentation. The best moisture content suitable for higher production of the antibiotic was found to be around 80% to 90% for rifamycin SV, B (Krishna *et al*., 1999; Venkateswarlu *et al*., 1999) and 70% for neomycin production (Vastrad and Neelagund, 2011). At 90% moisture level (Fig. 2), the production of rifamycin SV was about 212 mg/100 gds, similar condition reported with rifamycin B using wheat bran (Venkateswarlu *et al*., 1999). More or less availability of moisture content showed imbalance in porosity level between the substrate particles thereby decrease in rifamycin SV production was confirmed by less yield of the product. Different substrates with particles of approximately 1.0 mm in size to hold the moisture supplied, support the adherence, make nutrients available and exchange of oxygen for growth and product formation by the organism during fermentation were selected (Fig. 3). The substrate used gave a maximum yield of 217 mg/100 gds of rifamycin SV and alteration in substrate size decreased in product yield. Our results are in accordance to neomycin production using apple pomace, where a size of 1.2 mm of substrate showed favorable yield of the antibiotic neomycin (BM Vastrad and Neelagund, 2011).

The optimum initial pH for the production of rifamycin SV was found to be at 7.0 with a yield of 202 mg/100 gds (Fig. 4) considering it as an important parameter to be recognized during fermentations (Krishna *et al*., 1999). Whereas, lower and higher pH showed less rifamycin yield

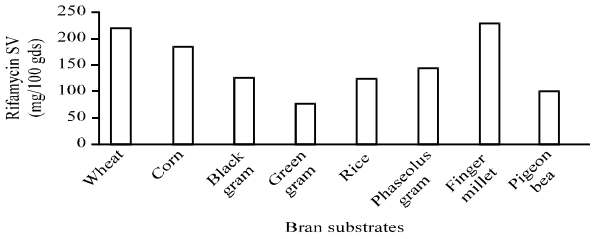

Fig. 1: Effect of solid bran substrates on rifamycin SV production

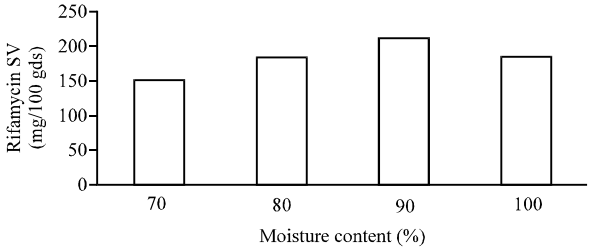

Fig. 2: Effect of initial moisture content on rifamycin SV production

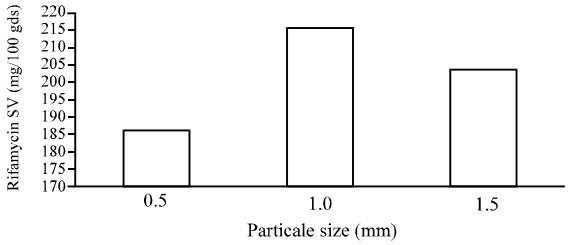

Fig. 3: Effect of particle size on rifamycin SV production

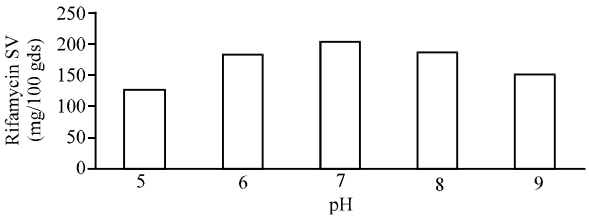

Fig. 4: Effect of pH on rifamycin SV production

due to poor growth of the organism in presence of unfavorable ion concentrations as it retards its growth during fermentation. Various incubation temperatures (25, 28, 30 and 32°C) were tested for maximum yield of rifamycin SV (Fig. 5). The organism though grew at various temperatures, the optimum incubation temperature for production of rifamycin was found to be at 30°C (210 mg/100 gds) (Nagavalli, 2009). According to reports on incubation temperatures used for rifamycin SV and B productions using different strains of *Amycolatopsis mediterranei*, 26, 28 and 30°C were optimum (Krishna *et al.*, 1999; Mahalaxmi *et al.*, 2010; Venkateswarlu *et al.*, 1999). This refers to the adaptation of the selected organism to its optimum conditions for its sustenance and product formation. Most of the antibiotic fermentations showed growth between 8 to 10 days of incubation time. The yield of rifamycin SV was found to be higher on 9th day of incubation, which showed 204 mg/100 gds (Fig. 6). On 8th day of fermentation, rifamycin SV yield was 196 mg/100 gds which slightly decreased on 10th (194 mg/100 gds) and 11th day (182 mg/100 gds) of incubation. Similar optimum incubation time of 9 days for fermentation was reported for metabolite production (Abd-Aziz *et al.*, 2008). Variation in over or less incubation time resulted in the decrease of rifamycin yield. Less incubation referring to improper metabolite formation and in over incubation less yield might be due to the exhaustion of sugars thereby resembling the horizon of stationary phase for the organism. A growth characteristic typically seen in secondary metabolite production where antibiotic production continued during both stationary and decline phase was demonstrated by El-Enshasy *et al.* (2007). Inoculum size also plays an important role in the production of rifamycin SV. Generally in rifamycin production, inoculum level of 20 to 40% was found to produce maximum antibiotic. Rifamycin SV production was found to be more at 30% inoculum level (215 mg/100 gds) (Fig. 7) reporting similar to rifamycin B production (Venkateswarlu *et al.*, 1999). Earlier, inoculum level of 40% was optimum for rifamycin SV and further increase or decrease in inoculum size showed fewer yields (Krishna *et al.*, 1999). However, it is made clear that sufficient amount of inoculum was essential for good growth and antibiotic yield by the organism.

In a fermentation process, the nutritional sources influence the overall output of the fermentation product. Glucose as a carbon source plays a vital role as a quick and an immediate source of carbon for the organism for its growth and antibiotic yield. Incorporation of glucose in the fermentation supported for yield of rifamycin SV from 230 to 246 mg/100 gds (Fig. 8). As reported earlier, indicating it to be a suitable carbon source for rifamycin fermentation under SmF and SSF conditions (Krishna *et al.*, 1999; Mahalaxmi *et al.*, 2010; Venkateswarlu *et al.*, 1999) and even in lovastatin production using *Monascus purpureus* (Sayyad *et al.*, 2007). (Oshoma and Ikenebomeh, 2005) have reported the supplementation of glucose with rice bran to give more biomass and increased yield of end product which correlates with our findings. Other carbon sources too supported towards the production of rifamycin SV but were less effective in yield

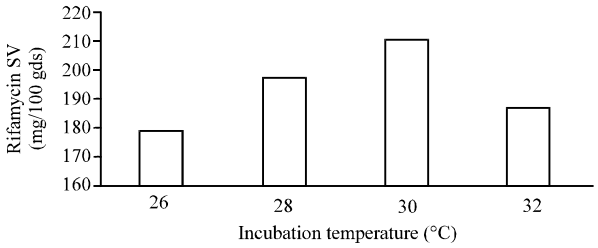

Fig. 5: Effect of temperature on rifamycin SV production

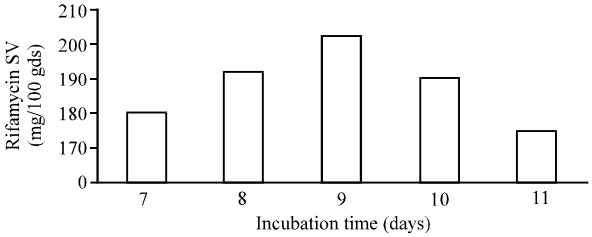

Fig. 6: Effect of incubation period on rifamycin SV production

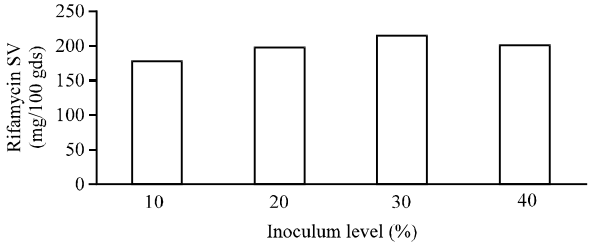

Fig. 7: Effect of inoculum size on rifamycin SV production

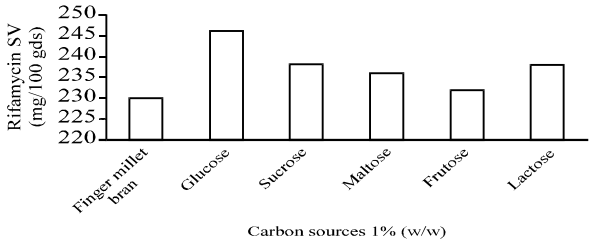

Fig. 8: Effect of carbon sources on rifamycin SV production

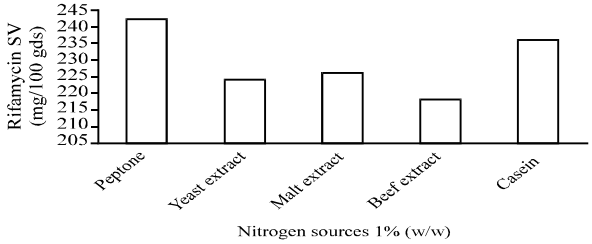

Fig. 9: Effect of nitrogen sources on rifamycin SV production

to glucose. Nitrogen sources availability during fermentation tend to determine the production of the metabolite. Peptone (244 mg/100 gds) and casein (236 mg/100 gds) showed an increase in the yield of rifamycin SV (Fig. 9). Whereas, other nitrogen sources like yeast extract, beef extract and malt extract showed minimal yield of the antibiotic indicating peptone and casein as efficient in support for more yield of cephamycin antibiotic (Bussari *et al*., 2008). Similar study conducted by Singh *et al*. (2011) using peptone as nitrogen source gave more amylase production. Yeast extract was not effective like peptone though it contains and provides essential co factors.

CONCLUSION

Cereal bran was evaluated for the production of rifamycin SV. The yield was found to be more with finger millet bran supplemented with glucose as additional carbon source when compared with other solid substrates. After optimization for solid state fermentation for various cultural and physiological conditions, the yield was 246 mg/100 gds of dry substrate of finger millet bran incorporated with glucose. The other favorable conditions include; incubation temperature of 30°C, pH at 7, inoculum level 30%, incubation time of 9 days and moisture content of 90%. Further studies for optimization and improvement are necessary for more rifamycin yield.

ACKNOWLEDGMENTS

The authors are grateful to the Curator, Microbial Genomics and Bio processing Research Unit, United States Department of Agriculture, ARS culture collection (NRRL) centre, Peoria for their kind supply of cultures used in this study.

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