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Stabilization of rifamycin-B oxidase from *Curvularia lunata*

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SUMMARY

The present investigation deals with the stabilization of rifamycin-B oxidase activity in the culture filtrate of *Curvularia lunata* using various methods. It was found that rifamycin-B oxidase activity in the culture filtrate was stable up to 5 days at 5 °C, degrading thereafter due to microbial contamination. The stabilization of enzyme activity was carried out by (i) concentration of culture filtrate and (ii) lyophilization, the activity remaining intact for 24 and 75 days, respectively. In another method, the enzyme activity was preserved by addition of 25–30% glycerol to the culture filtrate and the storability of enzyme activity then increased up to 90 days at 5 °C. The conversion of rifamycin-B to rifamycin-S using the stabilized rifamycin-B oxidase and fresh culture filtrate were comparable when run under similar conditions. The recovery of rifamycin-S powder from these experiments was not affected in any way in the presence of glycerol. Therefore, the present method of preservation of rifamycin-B oxidase may find industrial application for commercial production of rifamycin-S, which is an important intermediate for the synthesis of an antituberculosis drug, rifamycin.

INTRODUCTION

Among industrial applications of microorganisms, the production of rifamycin-S (RS) from rifamycin-B (RB) using rifamycin-B oxidase (RBO) is one of the most useful examples of microbial transformations. However, this biotransformation was made possible only after the discovery of microbial strains such as *Humicola* sp. ATCC 2060 and *Monocillium* sp. ATCC 2061 that produced RBO [1]. These organisms have low activities for rifamycin oxidation and RBO is intracellular. It requires energy intensive recovery steps such as cell disintegration and centrifugation to release enzyme for carrying out the desired transformation [2,3]. The chemical method [4] for the conversion of RB to RS, which was used earlier, required steps consisting of oxidation followed by hydrolysis. The yields were unsatisfactory for industrial application.

Scientists at the Institute of Microbial Technology (IMTECH) Chandigarh (India) have isolated an organism, *Curvularia lunata* var. *aeria* (MTCC 165) from soil that produces an extracellular RBO enzyme. The application of this extracellular enzyme for the conversion of RB to RS is limited due to its instability. This paper describes the work done to stabilize the activity of the enzyme in the culture filtrate (CF).

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MATERIALS AND METHODS

Production of RBO

The organism used in this study was *Curvularia lunata* var. *aeria*, obtained from the microbial type culture collection, IMTECH, Chandigarh (India), having identification No. MTCC 165.

The rifamycin-B oxidase (RBO) was produced in fermentation broth of *C. lunata* MTCC 165 grown in a suitable medium consisting of assimilable carbon and nitrogen sources. After harvesting, the mycelial mass was removed by filtration and the culture filtrate (CF) was used as a source of RBO in the present study. The stability of RBO enzymatic activity in CF stored at 5 °C for 6 days is shown in Table 1.

Methods of stabilization

In concentrated form. RBO was recovered from CF by a salting-out method using saturation levels (0–80%) of ammonium sulphate. After precipitation, the suspension was centrifuged at 15000 rpm for 30 min. The precipitate was dissolved in the minimum volume of phosphate buffer (0.1 M, pH 7.0) to yield a concentrated solution of RBO. The recovery of RBO from CF was around 86–88% (Table 2).

In powder form. The concentrated solution of RBO was dialyzed through cellulose dialyzer tubing (Arthur H. Thomas Co., Philadelphia, USA) against water to remove ammonium salts. The dialyzed enzyme solution after clar-

TABLE 1

Rifamycin-B oxidase activity in culture filtrate from *C. lunata* stored at 5 °C

Sl. No.	Days	Rifamycin-B oxidase activity (U/ml/h)		
		1st set	2nd set	3rd set
1	0	5.8	6.8	7.2
2	1	5.9	6.8	7.2
3	2	5.8	6.7	7.1
4	3	5.8	6.9	7.1
5	5	5.8	6.9	7.2
6	6	5.3	6.3	6.7
		5.73 ± 0.2268*	6.73 ± 0.2363*	7.083 ± 0.2037*

* Values represent as 95% confidence limit for the mean.

ification by centrifugation was lyophilized to yield a powder of partially purified RBO. The yield of stable enzyme in powder form was around 41% (Table 3).

Preservation of RBO in CF. To stabilize the activity of RBO in CF, various additives such as albumin, gelatin and glycerol were added and enzyme activity determined periodically (Table 4).

The stabilization studies in the present investigation were carried out at 5 °C, unless mentioned otherwise.

Enzyme assay

The assay of RBO in CF was based on the method reported in the literature [1]. Properly diluted CF was added to a pre-equilibrated RB solution and incubated at 50 °C for 1 h. After incubation, methanol-phosphate buffer (0.1 M, pH 6.5) in 1:1 ratio was added. The contents were kept in a boiling waterbath for 1 min to stop further reaction. The absorbance of RS was measured at 525 nm. The use of either deactivated enzyme or deactivation after completion of reaction in the control sets for RBO enzyme assay gave similar results. Hence, thereafter for RBO activity determination, the enzyme was deactivated after completion of reaction.

One unit of RBO was defined as the equivalent of 1 μmol RS formed in 1 h per ml of CF or per mg of powder enzyme assay conditions.

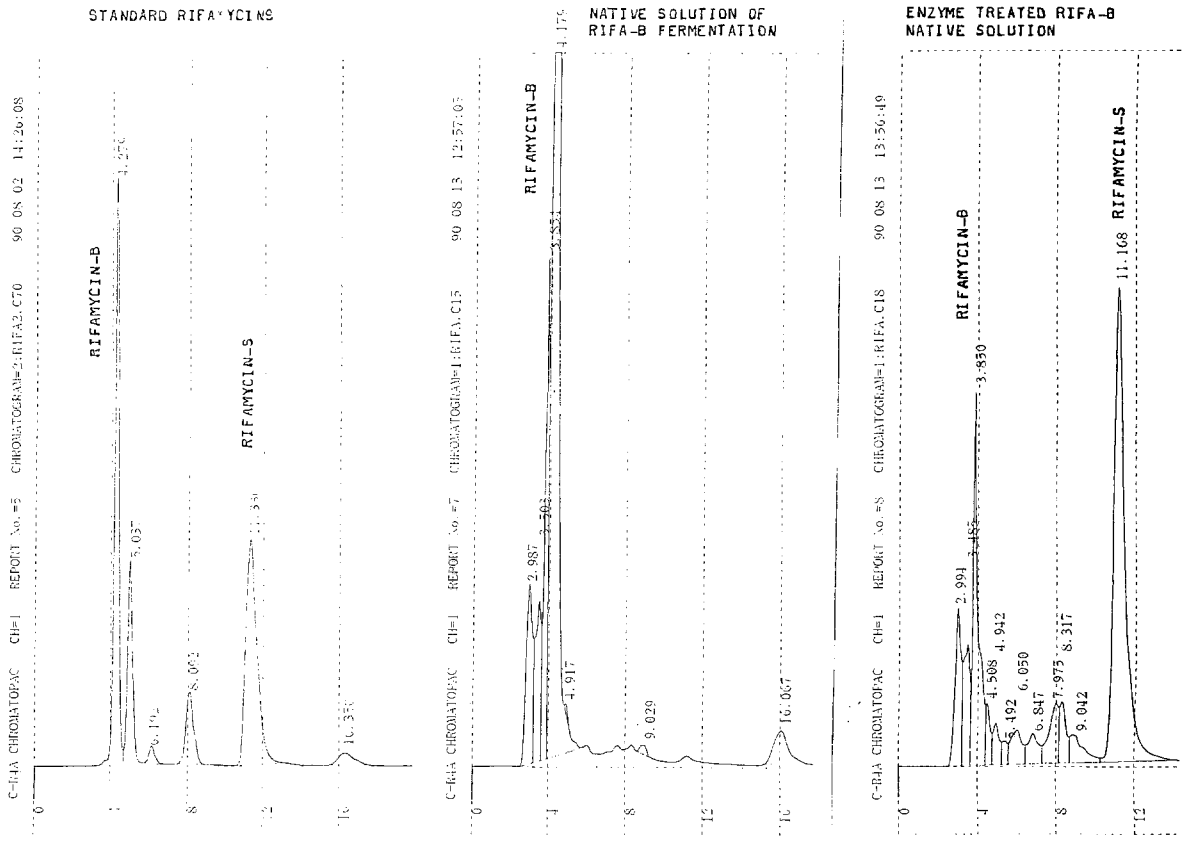

Fig. 1. HPLC chromatograms.

Conversion of RB to RS

The fermented broth containing RB produced by *Nocardia mediterranei* was filtered and transferred to a reaction vessel; to this RBO was added for conversion of RB to RS. The reaction was carried out with stirring at optimum temperature until the residual concentration of RB was almost nil.

The progress of the enzymatic reaction was followed by high-performance liquid chromatography in the Shimadzu system using an ODS C_{18} column at 254 nm and mobile phase of a mixture of ammonium formate buffer and methanol [5]. A typical chromatogram is shown in Fig. 1.

After the reaction was over, residual RB and RS formed were estimated at 425 and 525 nm, respectively, by spectrophotometric methods [6].

RESULTS

Storability of concentrated and powder RBO

Stability of concentrate. The activity of RBO in concentrated solution remained more or less constant up to 24 days (Table 2).

Stability of lyophilized powder. It was found that RBO activity could be preserved for up to 60 days (Table 3).

Stability of RBO in preserved CF. Two stabilizers, i.e., albumin and gelatin, were added to CF at 0.1 and 1% concentrations. RBO activity was determined periodically for 6 days (Table 4). It was found that the activity of RBO was considerably reduced on the 6th day of storage using albumin and gelatin as stabilizers in the culture filtrate. In another experiment (data not presented), where glycerol was used as stabilizer at 33.3 or 46% concentrations in the CF, the enzyme activity remained more or less constant during a storage period of 6 days without any contamination problem.

TABLE 2

Rifamycin-B oxidase activity in ammonium sulphate precipitated concentrate[a] stored at 5 °C

Sl. No.	Days	Rifamycin-B oxidase activity (U/ml/h)		
		1st set	2nd set	3rd set
1	0	62.3	31.1	67.1
2	6	63.4	29.8	67.5
3	12	66.7	33.5	66.0
4	18	63.0	32.8	67.1
5	24	62.3	32.8	67.1
		63.54 ± 1.76*	32.0 ± 1.881*	66.96 ± 2.214*

[a] From culture filtrate, rifamycin-B oxidase in concentrated solution was obtained by the salting out method using 0–60% ammonium sulphate saturation.

* Values represent as 95% confidence limit for the mean.

TABLE 3

Rifamycin-B oxidase activity in lyophilized powder stored at 5 °C

Sl. No.	Days	Rifamycin-B oxidase activity (U/ml/h)	
		1st set	2nd set
1	0	1.467	1.377
2	18	1.437	–
3	29	–	1.377
4	32	1.437	–
5	47	1.737	1.587
6	60	1.437	1.320
		1.49 ± 0.020*	1.45 ± 0.019*

* Values represented as 95% confidence limit for the mean.

In order to determine the optimum concentration of glycerol to stabilize RBO, varying concentrations of glycerol were added to the culture filtrate to give concentrations of glycerol of 10, 15, 20, 25 and 30% in the CF. The enzyme activity was determined periodically for 120 days (Table 5).

Glycerol at a concentration of 10% could stabilize RBO for up to 28 days. After that the enzyme activity was reduced due to heavy microbial contamination. Glycerol at 15 and 20% concentration stabilized RBO for up to 35 days, after which CFs were found to be contaminated with microbial growth. The activity of RBO could be stabilized at 25 and 30% concentrations of glycerol for 90 days and CFs were free from contamination.

Enzymatic conversion of RB to RS

The conversion of RB to RS was carried out on a laboratory scale, using RBO in concentrated form, powder form and glycerol stabilized CF and, compared with conversion using fresh CF of *C. lunata*. In all three forms of the enzyme, the conversion of RB to RS was comparable when parameters of conversion were kept absolutely identical. It was found that one unit of enzyme converted 2500 units of RB in 6 h, and that the presence of glycerol did not affect the recovery process of RS.

DISCUSSION

Generally, enzymes in solution are more stable at 0–4 °C than at higher temperatures. Further, liquid enzyme preparations are less stable compared to powder preparations stored in the dark at low temperatures [7]. RBO produced by *C. lunata* is found to be stable at 4–5 °C in all the four forms used, as indicated earlier.

TABLE 4

Rifamycin-B oxidase activity in culture filtrate with stabilizers stored at 5 °C

Sl. No.	Stabilizer	Concentration of stabilizer (%)	Rifamycin-B oxidase activity (U/ml/h) on day				
			0	1	2	5	6
1	Albumin	0.1	7.6	7.6	8.0	7.8	4.9
		1.0	7.8	7.6	8.2	7.6	5.9
2	Gelatin	0.1	7.6	7.6	7.6	7.6	5.3
		1.0	7.8	7.2	7.4	7.6	5.1
3	Control*		7.6	7.6	7.6	7.6	6.2

* Control values represent untreated culture filtrate.

TABLE 5

Storability of rifamycin-B oxidase in culture filtrate with varying concentrations of glycerol at 5 °C

Sl. No.	Days	Rifamycin-B oxidase activity (U/ml/h) of various concentrations of glycerol in percent				
		10[a]	15[b]	20[b]	25[c]	30[c]
1	0	6.22 ± 0.5416	6.20 ± 0.5413	6.17 ± 0.5365	5.95 ± 0.4609	5.87 ± 0.4389
2	7	6.27 ± 0.5421	6.25 ± 0.5512	6.29 ± 0.5310	6.11 ± 0.5010	5.93 ± 0.4658
3	14	6.27 ± 0.5384	6.24 ± 0.5567	6.27 ± 0.5383	5.95 ± 0.4568	5.95 ± 0.4604
4	21	6.27 ± 0.5421	6.24 ± 0.5567	6.27 ± 0.5383	6.10 ± 0.5339	6.03 ± 0.4696
5	28	6.26 ± 0.5358	6.24 ± 0.5567	6.24 ± 0.5567	6.10 ± 0.4825	6.03 ± 0.4696
6	35	5.28 ± 0.4098	6.24 ± 0.5567	6.17 ± 0.5220	5.95 ± 0.4603	5.95 ± 0.4606
7	42	4.71 ± 0.4425	6.11 ± 0.5260	6.11 ± 0.5220	5.95 ± 0.4603	5.95 ± 0.4606
8	60	4.67 ± 0.4593	6.11 ± 0.5260	6.11 ± 0.5220	5.99 ± 0.4786	5.92 ± 0.4419
9	80	4.34 ± 0.6369	6.09 ± 0.5371	6.05 ± 0.5372	5.99 ± 0.4603	5.95 ± 0.4568
10	90	3.88 ± 0.4846	6.05 ± 0.5329	6.11 ± 0.5267	5.99 ± 0.4786	5.87 ± 0.4420

[a] Culture filtrate having 10% glycerol concentration showed heavy contamination.
[b] With glycerol concentration of 15 and 20% culture filtrate was also contaminated.
[c] Culture filtrates with 25 or 30% glycerol were free from contaminations.
Values given represent mean ± s.d.; $n = 3$.

Several additives, such as enzyme substrates, metal ions, salts, anions, polymers, sugar and glycerol, have been used as enzyme stabilizers, and among these ammonium sulphate is routinely used to stabilize enzymes [8]. The present study demonstrates that RBO from *C. lunata* could also be stabilized by ammonium sulphate for a period of 24 days at 5 °C.

Glycerol and other antifreezers at varying concentrations have frequently been used to stabilize enzymes like aldehyde dehydrogenase [9], protease [10], ribonuclease [11], fungal amylases [12], glucoamylase [13], and chymotrypsin [14]. RBO produced by *C. lunata* has been found to be stabilized by the addition of 25–30% glycerol in CF and the enzyme activity could be preserved for more than 90 days at 5 °C.

The stabilization mechanism of glycerol is not clearly understood although glycerol is known to maintain native protein conformation [7], and the storability of enzymes in glycerol is certainly influenced by the repression of microbial growth due to its high osmotic pressure [10].

Bovine serum albumin and other soluble proteins such as gelatin, are also reported to stabilize enzymes [15]. RBO activity in the CF of *C. lunata* could not be stabilized beyond 6 days either by using albumin or gelatin.

The conversion of RB to RS using stabilized RBO preparations and fresh culture filtrate was comparable on a laboratory scale under identical conditions, and the recovery of RS was 65–70%. Moreover, the recovery process was not affected by the presence of glycerol.

On an industrial scale, the use of concentrated or powder forms of RBO may not be an economically viable proposal for the routine conversion of RB to RS, due to high cost of enzyme production. The cost of RBO production may be considerably reduced if enzyme-rich cul-

ture filtrate could be used for the conversion. However, instability of enzyme in CF is a major limiting factor.

Our studies, showing that RBO enzymatic activity in the culture filtrate can be stabilized with glycerol, offer an excellent solution to these problems. The stabilized CF containing RBO has many advantages. They include: (i) the storability of (stabilized) RBO in CF for more than 90 days at 5 °C; (ii) freedom of CF from contamination throughout its storage period; (iii) the fact that the stored stabilized RBO may be used as and when necessary, and the use of fresh CF of RBO for conversion of RB to RS is not a requirement now; (iv) the conversion of RB to RS was comparable to that of other stabilized RBO preparations including fresh CF with recoveries of RS around 70%; and (v) the recovery process of RS was not affected by the presence of glycerol.

Therefore, the present investigation is of great commercial significance for the enzymic conversion of RB to RS.

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