**Introduction**

Cholesterol (5-cholesten-3β-ol) is found in all body tissues, especially in the brain, spinal cord and as component of cell plasma membranes. It is necessary for the human body in small amounts; however, a high blood cholesterol level increases the risk of heart diseases, such as coronary heart disease, arteriosclerosis and other clinical disorders (Lehninger et al., 2005). It is also a constituent of animal foods such as eggs, meat and dairy products, determination of which in food industries is of primary importance for quality control and to select a diet for low intake of cholesterol (MacLachlan et al., 2000).  

Cholesterol oxidase (COD; EC 1.1.3.6), is a bifunctional enzyme, which catalyzes the oxidation of cholesterol (5-cholesten-3-ol) to the temporary intermediate 5-cholesten-3-one with the reduction of molecular oxygen to hydrogen peroxide, and the isomerization of the Δ5-bond to Δ4-bond (4-cholesten-3-one) (Salva et al., 1999; Yamashita et al., 1998). It was observed that there is an increase in cardiovascular diseases and cardiac arrest is a major cause of death all over the world (Davisa et al., 1995). An increasing need for specific estimation of cholesterol in clinical samples has enhanced the importance and demand of COD in the pharmaceutical industry, because assays incorpo-
rating COD are extremely simple, specific, highly sensitive, rapid, cost effective, and reproducible and thus offer distinct advantages over the conventional Liebermann-Burchard analytical methodologies which employ corrosive reagents and can be prone to unreliable results due to interfering substances such as bilirubin, creatinine, etc. (Allain et al., 1974). In recent years, researchers have made efforts to develop cholesterol biosensors based on COD, which enables rapid and economic estimation of serum and food cholesterol levels without interference with other substances, by means of simple disposable test kits (Basu et al., 2006; Kumara et al., 2000; Özer et al., 2007; Singh and Pundir, 2003; Singh et al., 2006; Vidal et al., 1999).

COD is a low-yield, high-value product. The commercial culture media require the addition of an expensive inducer such as cholesterol to increase the yield (Lee et al., 1997, 1999; Yazdi et al., 2001). Increasing the yield with cost-effective media is major goal for this enzyme. The enzyme is also being used in the production of precursors of hormonal steroids from cheaper 3-hydroxy steroidal nucleus (Fujishiro et al., 2002; Isobe et al., 2003). COD from Streptomyces sp. is also having agricultural importance, due to its insecticidal activity against insects like boll weevil larvae (Greenplate et al., 1995). It also shows excellent stability as well as rigio-, stereo- and enantioselective oxidation in organic solvents and detergents at high concentrations (Isobe et al., 2003).

The conventional method of medium optimization involves varying one parameter at a time and keeping the others constant. This method is extremely time consuming and often does not bring about the effects of interaction among the various parameters. To overcome this difficulty, RSM can be employed to optimize the medium components (Balusu et al., 2005). RSM and factorial design are important tools to study the effects of both the primary factors and their mutual interactions on intracellular carbonyl reductase to determine the optimal process conditions. Experimental designs for optimization have been commonly used for the optimization of multiple variables with a minimum number of experiments (Kalil et al., 2000).

Materials and Methods

Media components and chemicals. All media components, viz., glucose, sucrose, fructose, maltose, xy- lan, galactose, starch, lactose, soyabean meal, peptone, yeast extract, malt extract, meat peptone, and beef extract used in the study were purchased from Hi-media Laboratories Limited, Mumbai, India. Glycerol, magnesium sulphate, NaCl, ammonium sulphate and ammonium nitrate were purchased from S.D. Fine Chemicals, Mumbai, India. Horseradish peroxidase was purchased from Sisco Research Laboratory, Mumbai, India. o-Dianisidine dihydrochloride was purchased from Sigma Chemical Co., Bangalore, India.

Microorganism and growth medium. Streptomyces lavendulae NCIM 2421 and S. lavendulae NCIM 2499 used in the present study were obtained from National Collection of Industrial Micro-organisms (NCIM), National Chemical Laboratory, Pune, India. The slants were maintained on MGYP (Malt extract 3.0 g/L, glucose 10.0 g/L, yeast extract 3.0 g/L and peptone 5.0 g/L) media. Subcultured actinomycetes were incubated for 7 days at 30°C and stored at 4°C and subcultured every 20 days in the above-mentioned media. The production medium of COD contained (g/L) soluble starch, 15; yeast extract, 4; malt extract, 2; peptone, 5; K2HPO4, 1; MgSO4·7H2O, 0.5; NaCl, 0.5. The pH of the medium was maintained at 7.0 ± 0.05 before autoclaving. The seed medium was same as that of production medium, with soluble starch at 10 (g/L) (Varma and Nene, 2003).

Fermentation studies. Ten milliliters of saline solution (containing 0.1% tween 80) was added in to the growing slants and scraped to release the spores. From slant suspension, 2 ml of spores (containing 10⁷ spores/ml) were transferred to 50 ml of sterile seed medium in 250 ml Erlenmeyer flask and incubated at 30 ± 2°C for 48 h at 180 rpm. Fermentation was carried out in 250 ml Erlenmeyer flask, each containing 50 ml sterile production medium. The medium was inoculated with 10% (v/v) of 48 h-old seed cultures. The inoculated flasks were incubated on rotary shaker at 30 ± 2°C for 72 h at 180 rpm.

Optimization of fermentation medium components using one-factor-at-a-time method. The one-factor-at-a-time approach was used to investigate effects of medium components like carbon, nitrogen, mineral sources and environmental factors such as pH, on biomass and COD production. It involves changing one independent variable while fixing all the others at a certain level. Various media components/parameters have been optimized in the present research by using this technique.

Effect of seed age and seed inoculum size: To deter-
mine the optimal seed age for COD production, this organism was cultivated at various seed ages (12, 24, 36, 48, 60 and 72 h). The effect of seed inoculum size on biomass and COD production was investigated, by varying the seed inoculum size from 4 to 14% v/v.

Effect of carbon sources: The influence of different carbon sources on COD production was investigated by substituting soluble starch of basal medium with different carbon sources viz., xylan, sucrose, maltose, fructose, glucose, galactose, glycerol, lactose and cholesterol at a concentration of 1.5% w/v.

Effect of nitrogen sources: To investigate the effect of nitrogen sources on COD production, cells were cultivated in the medium containing various organic and inorganic nitrogen sources, including beef extract, meat peptone, peptone, soyabean meal yeast extract, ammonium sulphate and ammonium nitrate. In the study, yeast extract and peptone present in basal production medium at a concentration of 0.4% (w/v) and 0.5% (w/v), respectively, were replaced with different nitrogen sources, at a concentration of 0.9% (w/v).

Effect of initial pH: In order to investigate the effect of initial pH on biomass and COD production, Streptomyces lavendulae NCIM 2499 was cultivated with different initial pH (5.0–8.5) in shake flask cultures.

Fermentation profile study: The effect of fermentation time on pH, COD and biomass production was investigated. The flasks were harvested from 24–120 h at time intervals of 12 h and analyzed for the said parameters.

Analytical methods. Enzyme assay: COD activity was determined spectrophotometrically by measuring the increase in absorbance of o-dianisidine(oxidized) at 500 nm. The reaction mixture contained 2.7 ml of 0.01% o-dianisidine in potassium phosphate buffer (pH 7.5), 0.1 ml of cholesterol solution (0.5% w/v of cholesterol with 10% Triton X-100 in deionized water), and 0.1 ml of horseradish peroxidase (100 U/ml). To that reaction mixture 0.1 ml of cell free extract (0.1–0.2 U/ml of COD) was added (Masurekar and Goodhue, 1978).

\[
\text{cholesterol} + \text{O}_2 \rightarrow 4\text{-cholesten-3-one} + \text{H}_2\text{O}_2
\]
\[
\text{H}_2\text{O}_2 + \text{o-dianisidine (reduced)} \rightarrow 2\text{H}_2\text{O} + \text{o-dianisidine (oxidized)} \quad \text{(intense red color)}
\]

Conditions: pH 7.5, temp. 25°C, path length 1 cm, A₅₀₀. Calculations:

\[
\text{Enzyme activity (U/ml)} = \left( \frac{\Delta A_{500\text{nm}}/\text{min(test)} - \Delta A_{500\text{nm}}/\text{min(blank)} \times 3 \times d.f.}{7.5 \times 0.1} \right)
\]

Where,

\[
d.f. = \text{dilution factor}
\]

\[
7.5 = \text{molar extinction-coefficient of o-dianisidine(oxidized) at 500 nm at 25°C}
\]

\[
0.1 = \text{vol. of COD enzyme sample}
\]

\[
3 = \text{volume of assay (in ml)}
\]

Biomass estimation: For dry weight determinations, the cells were recovered by centrifugation of fermentation broth at 8,000 rpm (3,541 x g) for 20 min and washed twice with distilled water. The recovered biomass was dried to a constant weight at 80°C for 24 h.

Optimization of fermentation medium using the statistical designs. To examine the interactions and optimize the concentrations of production medium optimized by the one-factor-at-a-time approach, two statistical approaches, orthogonal array (Taguchi’s design) and RSM, were carried out to get maximum yield of COD. Experimental designs and analysis of experimental data were performed by using two software programs MINITAB 13.30 and Design-Expert Version 6.0.10, Stat-Ease Inc., Minneapolis, USA.

L₁₂-orthogonal array design: Taguchi’s orthogonal array provides an alternative to standard factorial designs. Taguchi constructed a special set of general design guidelines for factorial experiments that cover many applications. In this method, a special set of arrays called orthogonal arrays is used which stipulates the way of conducting the minimal number of experiments and providing complete information on all the factors that affect the performance of experiments. Factors and interactions are assigned to the array columns via linear graphs. It also provides a powerful and efficient method for designing products that operate consistently and optimally over a variety of conditions (MINITAB user’s guide 2, home page: http://www.itl.nist.gov/div898/handbook/pri/section5/pri56.htm). The major advantage of orthogonal array design is that it can be designed for more than two levels.

The medium that resulted in the highest enzyme titer with the one-factor-at-a-time approach was considered the basal medium and significant variables were identified by L₁₂-orthogonal array design. The six media variables used for the design were glycerol, soyabean meal, malt extract, K₂HPO₄, MgSO₄ and NaCl. Each variable was represented at two levels, high and low. The full experimental plan with respect to their coded forms is listed in Table 1.
obtained from the orthogonal array experiment were selected for optimization at the higher level, in which the various concentrations of the selected media components were optimized and interactions among them were investigated, to produce the maximum COD titer. The four independent variables, viz. soyabean meal (A), malt extract (B), \( \text{K}_2\text{HPO}_4 \) (C), and NaCl (D), were studied at five different levels, by experimental plan, in a set of 21 experiments. The minimum and maximum ranges of variables were investigated; a full experimental plan with respect to their values in actual form is listed in Table 2.
Results and Discussion

Fermentation optimization using one factor at a time (Classical Method)

Optimization of seed inoculum size and seed age: Maximum COD production (1.14 U/ml) was achieved when 6% v/v inoculum size was employed (Fig. 1); further increase in inoculum size showed decrease in COD activity, whereas biomass production was increased. In the study of seed age optimization, the maximum yield of COD was found when production medium was inoculated with seed of 48 h (1.28 U/ml) (Fig. 2). Seed of 12, 24, and 36 h have given low COD as well as biomass production, which may be due to the low cell density; seeds of 60 and 72 h have also given low productivity, which may be due to the aging of cells (Stanbury, 1997a).

Effect of carbon source: Figure 3 shows the effect of different carbon sources on COD production. Among the carbon sources screened, soluble starch and glycerol supported the maximum COD production as 1.09 U/ml and 1.23 U/ml respectively, at 1.5%, after 72 h fermentation. All carbon sources supported the growth of microorganism except cholesterol, which may be because S. lavendulae is a constitutive producer of COD (Varma and Nene, 2003). Lee et al. (1997) evaluated various carbon sources on Rhodococcus equi no. 23 and found cholesterol as inducer of COD at 0.1% w/v. Lee et al. (1999) in another study, evaluated different concentrations of cholesterol (0.52–1.08% w/v), on R. equi no. 23, and concluded that maximum productivity of COD as 0.24 U/ml, was obtained at 0.918% w/v. However, Yazdi et al. (2001), working with Rhodococcus equi 2C, also found cholesterol as a best inducer at 0.15% w/v. In another study, Kreit et al. (1994) evaluated several fatty acids on Rhodococcus sp. GK1, for COD production; they concluded that hexanoate as sole carbon source gave a maximum COD production of 100 U/g DW.

Effect of nitrogen sources: Different organic and inorganic nitrogen sources were evaluated for their effects on COD production and biomass growth as shown in Fig. 4. All organic nitrogen sources gave promising results, whereas inorganic nitrogen supported very low biomass and COD production. Mixtures of yeast extract and peptone (control) and soyabean meal gave highest production of 1.23 and 1.21 U/ml, respectively. It was because soyabean meal contains significant amounts of carbohydrates and fatty acids (Stanbury, 1997b); fatty acids were reported to enhance COD production (Kreit et al., 1994). Lee et al. (1997) worked on Rhodococcus equi no. 23 and reported yeast extract as the best nitrogen source at 0.4–0.5% w/v for producing COD. Rhodococcus equi 2C supported
maximum production of enzyme with yeast extract and diammonium hydrogen phosphate, at 0.18 and 0.09 U/ml, at 0.3 and 0.1% w/v, respectively (Yazdi et al., 2001).

**Effect of pH:** Figure 5 shows the effects of different initial pH values on DCW and COD production. An initial pH of 7.5 supported the maximum production of COD (1.21 U/ml) as well as biomass (1.2% w/v); the enzyme was also reported as most stable at that pH range (Varma and Nene, 2003). Yazdi et al. (2001) studied the effects of different initial pH on *Rhodococcus* sp.; and concluded pH 8 as optimal, whereas Lee et al. (1997) found initial pH of 7.0 as optimal for enzyme production from *Rhodococcus* sp. 2C.

**Fermentation profile study:** The effect of cultivation time on COD production under optimum conditions (Fig. 6) showed an optimum growth time of 72 h to be required for maximum total enzyme production. The enzyme production by *S. lavendulae* was observed as growth dependent, and the growth phase was completed at 72 h. The end of growth phase may have been due to depletion of nutrients in the medium (substrate limitation) or accumulation of some autotoxic products by the organism (Stanbury, 1997c); hence no further increase in enzyme production was observed. After 72 h, the enzyme activity decreased slightly, while the total dry matter remained stationary till 120 h. The pH adjusted to 7.5 initially was seen to decrease till 36 h (pH 5.57), then it increased drastically after 48 h, and remained almost constant after 72 h. Change in pH during the fermentation doesn’t have any effect on COD productivity (Varma and Nene, 2003).

**Media optimization by orthogonal array method**

An L₁₂-orthogonal matrix was used to investigate the most significant factors of medium components obtained from one factor at a time (Table 1). Table 3 shows the response for means and signal-to-noise ratio (larger is better). The last two rows in the tables show delta values and ranks for the system. Ranks and delta values help in assessing which factor having greatest effect on the response characteristics of interest. Delta measures the size of the effect by taking the difference between the highest and lowest characteristics average for a factor. A higher delta value indicates a greater effect of that component. Rank orders the factors from the greatest effect (on the basis of
Streptomyces lavendulae NCIM 2499

delta values) to the least effect on the response characteristics. The order in which the individual components affected the production of COD was malt extract > soyabean meal > K$_2$HPO$_4$ > NaCl > MgSO$_4$ > glycerol, suggesting that malt extract has the maximum effect and glycerol has the least effect on COD production.

In the present study, glycerol, K$_2$HPO$_4$ and NaCl shown maximum production at level 1, whereas soyabean meal, malt extract and MgSO$_4$ have shown effect at level 2. These levels also represented the optimum concentrations of individual components in the medium. The predicted optimum concentrations were validated and a maximum production of 1.50 U/ml was obtained as compared to 1.30 U/ml by one factor at a time, after 72 h.

**Optimization of fermentation medium using response surface methodology**

Various media components evaluated as significant by the L$_{12}$-orthogonal array approach were further selected for higher level optimization of their concentrations by CCRD of RSM. The four most significant factors obtained form L$_{12}$-orthogonal array for COD production were malt extract, soyabean meal, K$_2$HPO$_4$ and NaCl. The experimental design and results of RSM for studying the effects of four independent variables are presented in Table 2. Based on a regression analysis of the data from CCRD experiments, the effects of four independent variables on COD production were predicted by a second-order polynomial equation as:

$$Y = + 1.68 - 0.48 \times A - 0.22 \times B + 0.014 \times A^2 - 0.10 \times B^2 - 0.094 \times C^2 - 0.094 \times D^2 - 0.17 \times A \times B - 0.041 \times A \times C + 0.064 \times A \times D - 0.36 \times B \times D$$

The coefficient of determination ($R^2$) for COD production was calculated to be 0.9992. The $R^2$ value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The $R^2$ value is always between 0 and 1. The closer the $R^2$ value is to 1.00, the stronger the model is and the better it

<table>
<thead>
<tr>
<th>Levels</th>
<th>Mean</th>
<th>S/N</th>
<th>Mean</th>
<th>S/N</th>
<th>Mean</th>
<th>S/N</th>
<th>Mean</th>
<th>S/N</th>
<th>Mean</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.91</td>
<td>-1.18</td>
<td>0.75</td>
<td>-2.76</td>
<td>0.72</td>
<td>-3.15</td>
<td>1.01</td>
<td>-0.44</td>
<td>0.86</td>
<td>-1.93</td>
</tr>
<tr>
<td>2</td>
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<td>-1.49</td>
<td>1.05</td>
<td>0.09</td>
<td>1.09</td>
<td>0.48</td>
<td>0.79</td>
<td>-2.23</td>
<td>0.95</td>
<td>-0.75</td>
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<tr>
<td>Delta</td>
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<td>0.3</td>
<td>0.30</td>
<td>2.85</td>
<td>0.37</td>
<td>3.63</td>
<td>0.22</td>
<td>0.79</td>
<td>0.09</td>
<td>1.18</td>
</tr>
<tr>
<td>Rank</td>
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<td>2</td>
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<td>3</td>
<td>5</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Where, A=glycerol; B=soyabean meal; C=malt extract; D=K$_2$HPO$_4$; E=MgSO$_4$; F=NaCl.

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**Table 4. ANOVA for response surface quadratic model.**

<table>
<thead>
<tr>
<th>Sum of squares</th>
<th>d.f.</th>
<th>Mean square</th>
<th>$F$-value</th>
<th>Prob &gt; $F$</th>
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</thead>
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<tr>
<td>Model</td>
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<td>14</td>
<td>0.21</td>
<td>510.35</td>
</tr>
<tr>
<td>A</td>
<td>1.28</td>
<td>1</td>
<td>1.28</td>
<td>3,119.93</td>
</tr>
<tr>
<td>B</td>
<td>0.28</td>
<td>1</td>
<td>0.28</td>
<td>683.68</td>
</tr>
<tr>
<td>C</td>
<td>9.67</td>
<td>1</td>
<td>9.67</td>
<td>2.35</td>
</tr>
<tr>
<td>D</td>
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<td>1.86</td>
<td>0.04</td>
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<tr>
<td>$A^2$</td>
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<tr>
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<td>0.15</td>
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<tr>
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<td>0.13</td>
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</tr>
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<tr>
<td>AC</td>
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<tr>
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<tr>
<td>BD</td>
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<td>1</td>
<td>0.44</td>
<td>1,070.85</td>
</tr>
<tr>
<td>CD</td>
<td>1.36</td>
<td>1</td>
<td>1.36</td>
<td>3.30</td>
</tr>
</tbody>
</table>
predicts the response. When expressed as a percentage, $R^2$ is interpreted as the percent variability in the response explained by the statistical model. It implied that the sample variation of 99.92% for COD production was attributed to the independent variables and only 0.08% of the total variation was not explained by the model. This ensures a satisfactory adjustment of the quadratic model to the experimental data. The ‘Pred $R^2$’ of 0.9047 is in reasonable agreement with the ‘Adj $R^2$’ of 0.9972. This indicated a good agreement between the experimental and predicted values for COD production. Table 4 reports the ANOVA for quadratic model of the experimental design. The model $F$-value 510.35 implied that the model is significant. Values of ‘Prob $> F$’ less than 0.0500 indicated that the model terms are significant. According to present model, factors A, B, A², B², C², D², AB, AC, AD, BD are significant model terms.

The 3D response surface and the 2D contour plots described by the regression model are drawn to illustrate the effects of the independent variables, and interactive effects of each independent variable on the response variable. The shape of the corresponding contour plots indicates whether the mutual interactions between the independent variables are significant or not. Each contour curve represents an infinite number of combinations of two test variables with the other two maintained at their respective zero levels. The elliptical nature of the contour in 3D response surface graphs (Fig. 7) depicted the mutual interactions of all the variables. There was a relative significant interaction between every two variables, and there was

![Fig. 7. 3D response plot for COD activity from *S. lavendulae* (U/ml).](image)

(a) The effect of K₂HPO₄ and malt extract on COD activity; (b) the effect of NaCl and malt extract on COD activity; (c) the effect of K₂HPO₄ and soyabean meal on antibiotic activity and (d) the effect of NaCl and K₂HPO₄ on COD activity.
a maximum predicted yield as indicated by the surface confined in the smallest ellipse in the contour diagrams.

Maximum COD was produced at 1.67 U/ml when all the variables were kept at their central values. The model predicted maximum COD production of 2.21 U/ml, which could be achieved using the medium (g/L) glycerol 10.0 ml/L, malt extract 20.0, soyabean meal 20.0, K$_2$HPO$_4$ 0.6, MgSO$_4$ 2.0 and sodium chloride 0.7. Thus, an overall 2.48-fold increase in COD was being predicted after validation of RSM.

The interactive effects of independent variables are depicted in Fig. 7. Among the four variables tested, K$_2$HPO$_4$ was shown to have minimal effects on response upon varying its concentrations from minimal to maximum level, whereas the contour curve clearly shows that on increasing the concentrations of soyabean meal and malt extract, there was significant decrease in response. It shows that soyabean meal and malt extract had inhibitory effects on COD production by increasing their concentrations. NaCl has also been shown to have minimal effects on response upon varying its concentrations at different levels. Therefore, NaCl and K$_2$HPO$_4$ were found to have minimal interactions with each other (which is evident from the relatively circular nature of the contour curve Fig. 7d), and other media components, whereas malt extract and soyabean meal were given maximum interactions with each other and other media components on increasing their concentrations (Fig. 7a, b, c).

Conclusion

The statistical methods, viz. orthogonal array and RSM, allowed exploring culture conditions and identification of key media ingredients for the production of COD with an overall increase by 2.48 fold. From an economic point of view the most important parameter for screening and optimization of media are time and cost. The combined strategy demonstrated advantages in comparison with traditional methods.

References


