IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
**Research Article**
November 2018 Vol.:13, Issue:4


# Solid-State Fermentation of *Tecomella undulata* Bark Using *Saccharomyces cerevisiae* and its Optimization Using Four Factor Box- Behnken Design

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
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**Submission:** 22 October 2018
**Accepted:** 28 October 2018
**Published:** 30 November 2018

HUMAN JOURNALS
**www.ijppr.humanjournals.com**

**Keywords:** *Tecomella undulata,* Solid State Fermentation, *Saccharomyces cerevisiae*, Response Surface Methodology (RSM), Box Behnken Design (BBD).

**ABSTRACT**

The aim of the study was to determine the effect of selected variables on fermentation of *T. undulata* bark. The bark was fermented at 30°C for 48h using a pure culture of yeast *Saccharomyces cerevisiae* and the method was optimized using response surface methodology (RSM). The independent variables, pH, inoculum volume (ml), temperature (°) and fermentation time (h) were optimized using four factor Box-Behnken Design to obtain a good amount of Lapachol after Solid State Fermentation. The method yielded a quadratic polynomial equation to predict the effect of independent variables on selected response. The experimental values agreed closely with the predicted values and the analysis of variance signified a good model fit. The Point prediction of the design expert software was used to determine the optimum conditions found to be temperature 47℃, Time 90 h, pH 7 and inoculum volume 11ml to produce maximum yield for Lapachol. Upon application of RSM "an adequate precision" of 5.508 was indicative of good validity of the model since ratio of greater than 4 is desirable. *T.undulata* bark was fermented effectively by a pure culture of *Saccharomyces cerevisiae* in absence of jaggery in a short span of time 90h as compared to conventional fermentation. The study also reports the presence of Lapachol in fermented extract of *T. undulata* bark which is used as an anticancer, antibacterial. The purpose of this study is to provide an overview of the study of production of known bioactive compounds with respect to Lapachol by biotransformation using Solid state fermentation.

## INTRODUCTION

Fermentation is prescribed as a method for drug preparation in Ayurveda for a wide variety of therapeutic purpose. The fermentation employed in Ayurveda is termed as 'Sadhan Kalpana' which involves chemical changes in the substrate through the activities enzymes released by microorganisms [1]. The ethanol produced during this process acts as a solvent as well as preservative [1]**.** Asavas (alcoholic medicaments prepared from powdered herbal drugs) and arishtas (alcoholic medicaments prepared from decoctions of herbal drugs) are the types of fermented Ayurvedic herbal drug preparations(1). In contrast to fermentation used in Ayurveda, Solid State Fermentation (SSF) is a technique which consists of the microbial growth and product formation on solid particles in the absence of water; however, substrate contains the sufficient moisture to allow the microorganism growth and metabolism[2]. SSF leads to higher yield and productivities or better product characteristics than traditional method for the cultivation of microorganisms under controlled conditions in the absence of free water. Wide range of microbes are used in the SSF system to produce economically diverse important products such as enzymes, organic acids, secondary metabolites, antibiotics, biofuels, biocontrol agents and vitamins at low cost and less labor[3]. Fermentation is important as fermented materials are easier to digest and assimilate the nutrients[4]. It retains enzymes, vitamins and other nutrients because it does not require heat. It also increases the nutritional value. It removes major fraction of sugars, making the components more bio-available[5]. In Ayurvedic preparations flowers of *Woodfordia fruticosa* are used for fermentation. In this study, we will apply solid state fermentation using a yeast strain *Saccharomyces cerevisiae* which can live in both aerobic as well as anaerobic conditions[6]. *T. undulata* seem. (Bignoniaceae) is traditionally known as Rohida and it is used as a hepatoprotective agent in various Ayurvedic formulations such as Rohitakarishta, Rohitakadyachurna, Rohitakaghrita and Rohitakalauha[7]. A wide range of pharmacological activities have been reported from *T. undulata* bark due to the presence of various chemical constituents[7]. Lapachol is a naturally occurring 1,4-naphthoquinone originally isolated from Tabebuiaavellanedae (Bignoniaceae) [8], used against, Trypanosome cruzi, cancer, viruses, bacteria, and Leishmania[9]. This constituent can also be found in other plant families such as, Verbenaceae, Leguminosae, Sapotaceae, Scrophulariaceae, Malvaceae and Proteaceae, however, its occurrence is higher in the Bignoniaceae family, particularly in the gender Tabebuia, along with other heterocyclic quinines[9]. In this investigation, fermented extracts of *T. undulata* bark were optimized using different optimization parameters by Response Surface Methodology. RSM is a collection of

mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables based on the polynomial equation to the experimental data, response which is influenced by several independent variables are optimized[10]. An experiment is a series of tests called runs, developed by BOX and collaborators in 50s and then migrated into modeling of numerical experiments[5]. Response surface methodology is a more advantageous multivariate statistical technique than the traditional single parameter optimization[4]. More specifically BBD is used in this regard. BBD is known to be more efficient than the three level full factorial RSM designs because it allows estimation of the parameters of the quadratic model, building of sequential designs, detection of lack of fit of the model and use of blocks[11]. Moreover, BBD has the desirable feature of needing the smallest number of experimental runs and it is useful in avoiding experiments performed under extreme conditions to provide unsatisfactory results[12]. The formulation Aristas is a traditional ayurvedic formulation which is currently being prepared using *W. fruticose* flowers and this process takes almost 35 days or more, also there is a risk of contamination in using flowers as an inoculum, as it contains other microbes also[13]. This experimental work shows optimization of fermentation process used in formulation of traditional herbal medicines using selected yeast strain (*S. cerevisiae*) in place of *Woodfordia fruticosa* flowers by means of RSM. Present study determines the effect of variables (substrate volume, temperature, pH and time) on selected responses using four-factor Box-Behnken Design.

## MATERIAL AND METHODS

Stem bark of *T. undulata* was collected from Lodhi garden, New Delhi. The samples were authenticated by National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India with ref no. NISCAIR/RHMD/Consult/2014/2472-51. The solvents used were from Merck (Darmstadt, Germany).

### Reference standard

Standard Lapachol were purchased from Sigma Aldrich fine chemicals.

### Strain

*S. cerevisiae* yeast strain was purchased from MTCC center, Institute of Microbiology Technology, Chandigarh, India.

**Revival of Culture**

A culture of *S. cerevisiae* was revived on yeast extract malt extract agar medium. The autoclaved medium was kept for 24 hours to get solidified and was streaked with lyophilized culture.

**Preparation of culture medium**

After 24h basal medium was prepared in two conical flasks of 250ml (10g dextrose, 1g peptone, 0.2g potassium nitrate, 0.2g ammonium dihydrogen phosphate, 0.005g magnesium sulphate, 0.01g calcium chloride in 100ml distilled water) adjusted to pH 6.0.

**Inoculation**

The autoclaved culture medium prepared above was inoculated with a loopful of actively grown slants and kept in shaker incubator at 30℃ for 48 h at150rpm(14).

**HPTLC analysis**

The above fermented extracts were filtered, concentrated and dried, the HPTLC quantification was carried out using toluene-ethyl acetate-glacial acetic acid (8.5:1.5:0.02v/v/v)(15) as a solvent system. The plates were scanned at 254nm, WINCATS software was used for recording and analyzing the chromatographic data[4].

**Preliminary trials**

The selected formulation is a fermented extract, so the factors considered were, pH of the solid medium, inoculum volume, temperature and fermentation time (Table No. 1). Since fermentation is an anaerobic process, so considering the volume of flask 250 ml (Erlenmeyer flask) the inoculum volume (ml) taken were 5, 6.5, 7 and 7.5 ml. The second factor was temperature ($^{O}$) 30, 32, 25 and 37. Third factor was time (h) 24, 36, 48 and 60. And fourth factor was pH of the solid medium 6, 6.5, 7 and 7.5.

**Experimental design**

Box-Behnken design (design expert software 10, trial version, Stat-Ease Inc., USA) A 4-factor, 3-level was applied to determine the best combination of variables for the desired responses [16]. In this investigation four process variables considered were A (temperature), B

(fermentation time), C (inoculum volume), and D (pH of solid medium). An experimental design of 29 runs containing 5 central points was made according to BOX-BEHNKEN response design for four selected parameters. The individual and interactive effects of these variables were studied by conducting the fermentation run at different levels of all factors.

| S. No. | pH of solid medium | Inoculum volume (ml) | Temperature ($^{O}$C) | Fermentation time (hour) |
|---|---|---|---|---|
| 1. | 6 | 5 | 30 | 24 |
| 2. | 6.5 | 6.5 | 32 | 36 |
| 3. | 7 | 7 | 25 | 48 |
| 4. | 7.5 | 7.5 | 37 | 60 |

## RESULTS

### Selection of variables

The process variables evaluated were inoculum volume, temperature, fermentation time and the pH of the solid medium, whereas the response was Lapachol content (µg/ml). *T. undulata* have been reported for various chemical constituent, however, fermentation has been known to biotransformation the chemical nature of substrate. The strain used in this study *S. cerevisiae* which is prevalent in wine and bakery industries [17]. HPTLC profiling shows that Lapachol was present in the fermented extract obtained after incubation for 48 h (Fig 2). The optimum conditions temperature 37℃, Time 60 h, pH 7.5 and inoculum volume 7.5 ml were found to be best after preliminary trials. These results were applied to RSM for further optimization. BBD was employed for the process which consisted 29 runs, each experimental run were analyzed during the software design expert 10 (Stat-Ease, USA) and fitted into multiple non-linear regression mode.

### Effect of variables on selected response

Figure 2 shows the 3D response surface plots, the result shows increase in temperature causes enhancement in the yield. Time also have a similar effect on values of response. The time duration for optimum Lapachol yield was 90 h. When the substrate volume was less than obtained value could not acquire the higher yield. Favourable conditions for observed to be temperature 47℃, Time 90 h, pH 7 and inoculum volume 11ml for Lapachol yield.

## Model fitting

The values of four variables, that is inoculum volume, temperature, pH of the medium and fermentation time with actual predicted Lapachol yield in 29 random experimental runs are shown in (Table No. 2). The quadratic polynomial model was used to correlate the independent variables with selected responses (Table No 3.). The best candidate to fit the data was the quadratic model after firt summary comparison. The predicted values correlate well with experimental values.

## Optimization

Upon application of RSM, an "an adequate precision" of 5.508 was indicative of good validity of the model since ratio of greater than 4 is desrirable. Based on model, optimum conditions were obtained using point prediction tool and the result observed were temperature 47℃, Time 90 h, pH 7 and inoculum volume 11ml for Lapachol content (Fig 4).

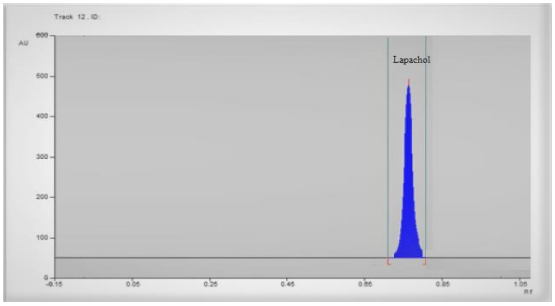


**Figure 1 HPTLC chromatogram of standard Lapachol**

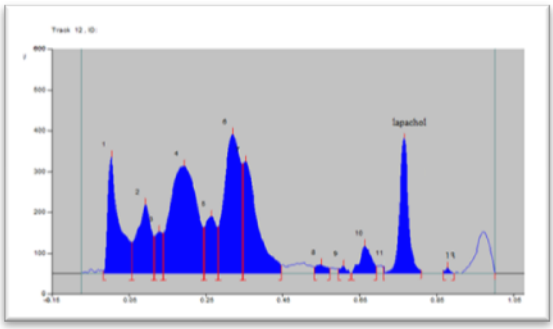


**Figure 2 HPTLC chromatogram of fermented extract of Lapachol**

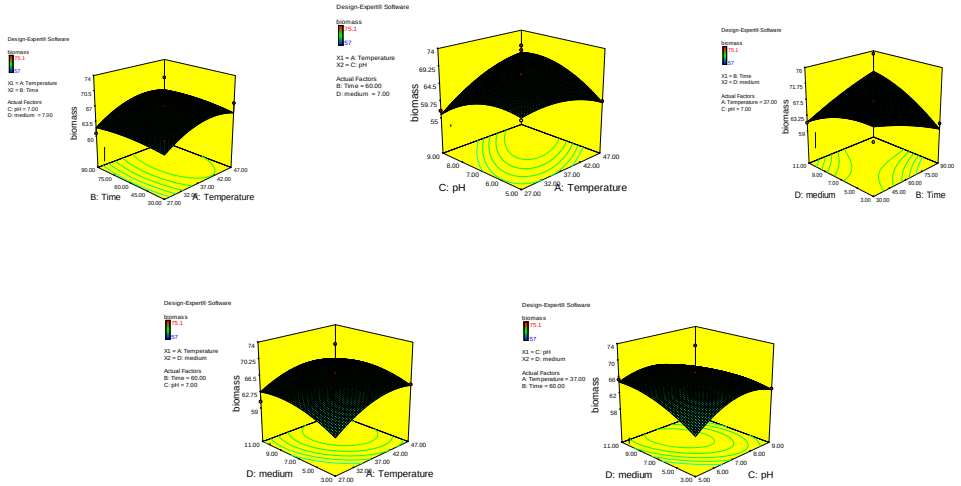


**Figure 2a: 3D Response Surface Plots Graphs Demonstrating Effects of Temperature, Time, pH And Inoculum Volume**

**Table No. 2 The Box- Behnken design with the independent variables**

| Run | A:Temp (C ) | B:Time (hrs.) | C:Inoculum volume (ml) | D:pH | Lapachol content (µg/ml) | Actual value | Predicted value |
|---|---|---|---|---|---|---|---|
| **1.** | 37 | 30.00 | 7.00 | 9.00 | **60.95** | 60.40 | 58.14 |
| **2.** | 37 | 60.00 | 7.00 | 7.00 | **61.1** | 61.99 | 60.96 |
| **3.** | 37 | 60.00 | 11.00 | 5.00 | **62.5** | 62.56 | 63.25 |
| **4.** | 37 | 90.00 | 3.00 | 7.00 | **57** | 58.15 | 60.08 |
| **5.** | 37 | 60.00 | 3.00 | 5.00 | **60.5** | 60.50 | 64.41 |
| **6.** | 27 | 30.00 | 7.00 | 7.00 | **69.4** | 69.70 | 71.36 |
| **7.** | 27 | 60.00 | 7.00 | 5.00 | **75.1** | 76.50 | 74.51 |
| **8.** | 27 | 60.00 | 7.00 | 9.00 | **67.79** | 67.40 | 65.15 |
| **9.** | 27 | 60.00 | 11.00 | 7.00 | **62.09** | 62.25 | 61.89 |
| **10.** | 37 | 90.00 | 7.00 | 5.00 | **66.08** | 67.60 | 64.79 |
| **11.** | 37 | 30.00 | 3.00 | 7.00 | **60.08** | 60.09 | 63.41 |
| **12.** | 37 | 90.00 | 11.00 | 7.00 | **60.7** | 59.30 | 60.16 |
| **13.** | 37 | 90.00 | 7.00 | 9.00 | **64.5** | 64.08 | 64.84 |
| **14.** | 37 | 30.00 | 7.00 | 5.00 | **62.08** | 62.08 | 65.18 |
| **15.** | 37 | 60.00 | 11.00 | 9.00 | **65.4** | 65.95 | 63.36 |
| **16.** | 37 | 60.00 | 7.00 | 7.00 | **67.08** | 67.50 | 67.25 |
| **17.** | 47 | 90.00 | 11.00 | 7.00 | **69.7** | 69.10 | 69.10 |
| **18.** | 37 | 60.00 | 3.00 | 9.00 | **59.7** | 59.70 | 59.92 |
| **19.** | 37 | 60.00 | 7.00 | 7.00 | **61.3** | 60.79 | 60.39 |
| **20.** | 37 | 60.00 | 7.00 | 7.00 | **62.8** | 62.40 | 63.22 |
| **21.** | 37 | 60.00 | 7.00 | 7.00 | **63.6** | 62.08 | 61.02 |
| **22.** | 27 | 90.00 | 7.00 | 7.00 | **73.56** | 73.00 | 72.65 |
| **23.** | 47 | 60.00 | 3.00 | 7.00 | **6s2.6** | 62.80 | 64.98 |
| **24.** | 47 | 60.00 | 7.00 | 5.00 | **59.7** | 60.70 | 61.58 |
| **25.** | 27 | 60.00 | 3.00 | 7.00 | **65.25** | 65.30 | 63.18 |
| **26.** | 47 | 30.00 | 7.00 | 7.00 | **64.99** | 64.08 | 63.18 |
| **27.** | 47 | 90.00 | 7.00 | 7.00 | **63.15** | 63.60 | 63.18 |
| **28.** | 47 | 60.00 | 7.00 | 9.00 | **61.4** | 61.80 | 63.18 |
| **29.** | 37 | 30.00 | 11.00 | 7.00 | **62.8** | 61.10 | 63.18 |

**Table No. 3 Analysis of variance (ANOVA) for the fitted quadratic polynomial model**

| SOURCE | SUM OF SQUARES | Df | MEAN SQUARE | F VALUE | P VALUE | |
|---|---|---|---|---|---|---|
| Model | 232.58 | 7 | 33.23 | 3.25 | 0.0164 | **SIGNIFICANT** |
| Lack of fit | 148.69 | 17 | 8.75 | 0.54 | 0.8334 | **NON SIGNIFICANT** |

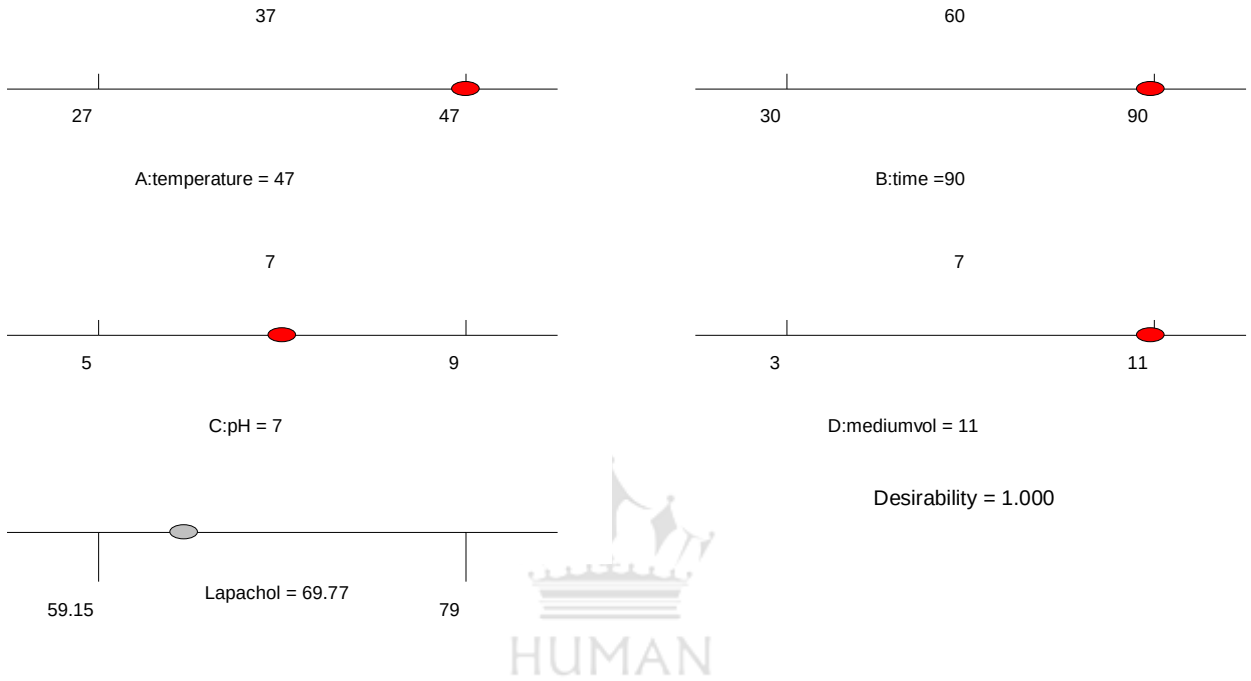


**Figure 3 Point prediction for design expert software**

## DISCUSSION

*W. fructicosa* is an integral component of all the asava/arista formulations mentioned in ancient texts. It has played the role of inoculum since time immemorial. *S. cerevisiae* is a yeast strain that can live in both aerobic and anaerobic conditions and it also inhibits the growth of other pathogenic fungi in the medium, therefore preventing contamination, therefore in this study, the process of traditional fermentation has been performed using a pure yeast strain and it was optimized using RSM. Firstly the bark was fermented at $30^0$ for 48 h using a pure culture of yeast *Saccharomyces cerevisiae*, after 48 h the HPTLC quantification was carried out using toluene-ethyl acetate-glacial acetic acid (8.5:1.5:0.02v/v/v) as a solvent system, HPTLC profiling shows that Lapachol was present in the fermented chloroform extract obtained after incubation for 48 h (Fig 2a). The optimum conditions temperature 37°C, Time 60 h, pH 7.5 and inoculum volume 7.5ml were found to be best after preliminary trials. These results were

applied to RSM for further optimization. which gave 29 runs, each experimental runs were analyzed during the software design expert 10 (Stat-Ease, USA) and fitted into multiple non-linear regression mode. The method yielded a quadratic polynomial equation and the experimental values agreed closely with the predicted values and the analysis of variance signified a good model fit (Table No. 3). The 3D response surface plots (Fig 2), shows increase in temperautre causes enhancement in the yield , when the substrate volume was less, than obtained value could not acquire the higher yield therefore the favourable conditions observed to be temperature 47℃, Time 90 h, pH 7 and inoculum volume 11ml that yielded 69.7µg/ml of Lapachol in fermented chloroform extracts. Present study indicates the importance of *S. cerevisiae* in determining the final composition of fermented extract and significance of selected variables in obtaining high yields of selected component. The product was fermented successfully and desired formulation was obtained in 90 h as compared to traditional method.

## CONCLUSION

Fermentation is an important process in traditional Indian medicine system and its standardization is critical for ensuring the quality of formulations. In this present study, the product was fermented successfully and desired formulation was obtained in 90 h. The process was optimized using a four factor Box-Behnken Design and the Point prediction of the design expert software was used to determine the optimum value of the four factors (fermentation parameters) for Lapachol content, indicating the significant effects of optimization after Solid-State Fermentation of the crude drug. In Ayurvedic preparations flowers of *Woodfordia fruticosa* are used for fermentation, but in this experimental work solid state fermentation was employed effectively using yeast strain *Saccharomyces cerevisiae* which produces the formulation in 90 h without contaminating the medium rather than 35 days mentioned in traditional method. This study also has reported the presence of Lapachol in fermented chloroform extract of *T. undulata* bark which is used as an anticancer, antibacterial. Therefore, this work has established that a yeast strain causes various changes in the chemical content of plant during fermentation which makes the compound more bioavailable.

## ACKNOWLEDGEMENT

The authors are grateful to the Department of Pharmacognosy and Photochemistry, School of Pharmaceutical Education and Research (SPER), Jamia Hamdard, New Delhi.

## AUTHOR CONTRIBUTIONS:

Richa Raj, designed, carried out and wrote up the research article, Vidhu Aeri, supervised and helped in designing the work, and proofread the research article.

## CONFLICTS OF INTEREST:

The authors declare no conflict of interest.

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