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Protein Rich Biomass Production by Novel Yeast *Cyberlindnera jadinii* MMS7 Using *Chara* sp. Hydrolysate and Evaluation of its Anti-Bacterial Activity

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ABSTRACT

A total of seven cultivable purified yeast phenotypes were isolated from soil collected from sugarecane bagasse dumping sites. By the genotypic characterization, new isolate MMS7 was confirmed as most promising yeast strain and it was identified as *Cyberlindnera jadinii* MMS7. We used this strain to investigate the feasibility of biomass production using hydrolysate of green *Chara* sp as sole medium. The ultrasonic pretreatment of the *Chara* sp biomass for hydrolyaste preparation was optimized as 0.35 WmL^{-1} (ultrasound power density) and 15 min (treatment time) using response surface methodology (RSM). The biomass of 15.05±0.12 g dwL^{-1} and protein concentration of 59.21±0.12% were obtained in *C. jadinii* MMS7, when cultivated in hydrolyaste. This study demonstrates the novel yeast *C .jadinii* MMS7 could be considered a promising single cell protein producing strain using *Chara* sp hydrolysate and it has high antibacterial activity against *Bacillus cereus*. Further, the exploitation of *Chara* sp. hydrolysate potential may use to aggregate value for algae cultivation.

Keywords: Biomass, *Cyberlindnera jadinii,* Hydrolysate, Pretreatment, *Chara* sp.

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INTRODUCTION

Beginning of the twentieth century, the microorganisms are used as a food and fodder ingredient for human and animals respectively. Among the microorganisms yeasts are easily acceptable than other microbes by the society for various applications because they have vast practical potential in biotechnology, particularly in wine and beer production. However, the yeasts are potential as a promising source of human and animal nutrition. They are also producing various metabolites like citric acid, ethanol, erythritol, industrial important enzymes etc. In addition, some types of yeasts have antimicrobial activities due to secrete toxins that can inhibit growth of mold and bacteria[1,2].

Among the yeasts *candida*, strain *Candida utilis* has gained wide attention because it fulfills the fodder yeast criteria due to high protein content in the dry cells. Besides, it has the ability to utilize various nutrients sources of the growth medium and rapid growth within short time[3]. Moreover, it has been approved by U.S food and drug administration (FAD) as safe for human consumption and included in the American Generally Recognized Safe List (AGRS). Therefore, yeast *C. utilis* biomass and its metabolites are widely used in the food industry and fodder production[4]. Many studies have shown that the *C. utilis* cell biomass contains large amount of essential amino acids, particularly lysine[5], varied types of vitamins B: riboflavin, folic acid, and biotin[6,7] and the steroid ergosterol[8]. Furthermore, this yeast also used for preparation of bioelements (protein and mineral - selenium, magnesium) that can be easily consumed by humans because they have the ability to bind with metal ions from the culture medium[9].

The yield and productivity of yeast biomass are entirely dependent on the physicochemical parameters of culture medium[10]. However, the degree of biomass production mainly depends on the nature of substrate and availability of substrates. Since, choosing the appropriate substrate is very important because they directly influence the fermentation products and cost of production. Nowadays, to reduce the cost of production the wastes are utilized as substrate for biomass production. However, the waste materials, need to

fulfill the following criteria of being abundant, nontoxic, regenerable and inexpensive. In addition to these, they should support the rapid growth of yeast cells with high-quality biomass. In other words, the organic wastes can be used as a substrate to reduce the total cost of biomass production and as an eco-friendly method of removing organic residues from wastes. However, the fermentation processes should be optimized for each type of substrate because the microbes are differently react with each substrate and thus, the rate of substrate utilization differs in each substrate[11].

The production of SCP from different microorganisms achieved by utilizing the residue of wheat straw, orange peel, sugarcane baggages, sweet orange, rice bran, paper mill wastewaters, cassava waste, sawdust, wood shavings, seed removed cobs of corn, sugar beet pulp, wastes of coconut, wastes from mango, wastes of grape, etc as substrate[11, 12]. However, at certain time the complex chemical characteristics of wastes limiting the bioconversion of the waste into biomass has to be hydrolyzed by any one of the physical method, chemical method, and biological method or combined methods before using them as substrates. In fact, pretreatment by previously mentioned methods increase the carbohydrates contents for microorganisms utilization1[11,13,14].

Many scientists have studied factors and chemical components of media favouring the growth of yeast. However, the use of *Chara* sp hydrolysate, to our knowledge, has not been investigated till now. Therefore, the aim of the present study was the use of hydrolysate prepared by ultrasonic pretreatment, from *Chara* biomass as nutrient medium for biomass production from selected isolated novel yeast strain and to evaluate its antibacterial activity.

MATERIALS AND METHODS

Materials

Yeast Peptone Dextrose (YPD) agar and other chemicals with highest purity or analytical grade used in this study were purchased from Himedia chemicals (Mumbai, India), Sigmae-Aldrich (Bommasandra, India) and Merck Chemicals Ltd.,(Mumbai, India).

Isolation of yeast strain

Soil samples were collected from different sugarcane bagasse dumping sites, Kancheepuram, Chennai, Tamil Nadu, India. Five gram of sieved sample was transferred into 100 mL of sterile distilled water and and then serially diluted until 10^{-5}. Spread plates were prepared using selective medium - saboraud dextrose agar (SDA) medium (Dextrose 40 gL^{-1}, Peptone 10 gL^{-1} and Agar 15 gL^{-1}, pH 5.6). To obtain pure cultures, the colonies with distinctive morphology were streaked on to separate SDA medium.

Chara sp. collection and crude extract preparation

The green alga *Chara* sp was collected from freshwater Lake (12.6620º N and 79.5435º E) Cheyyar , Tiruvannamalai District, Tamil Nadu, India and then thoroughly washed with tap water followed by distilled water to remove all debris. The cleaned *Chara* sp biomass was dried to a constant weight at 50 ºC in hot air oven. After drying, 1 kg of sample was grinded with 400 mL distilled water finally made into 1L. Filtered the extracts with Whatman No.1 filter paper and then used as a sole medium for screening the maximum biomass producing yeast strain.

Screening of maximum biomass producing yeast strain

All the isolates were screened for their ability to grown in the crude extract of *Chara* sp. The experiments were carried out in 250 mL Erlenmeyer flask using 100 mL of cured extract as a sole culture medium. Then the medium was inoculated with 10% v/v inoculum of each isolates and incubated in an orbital shaker incubator at 27 °C with pH 7.0 for 72 h under constant shaking at 120 rpm. At the end of the stationary phase, the culture of each isolate was used for biomass estimation. The maximum amount of biomass produced yeast strain was selected and used for further investigation.

Biomass estimation

The biomass was estimated by gravimetrical method. 10 mL of samples was added to pre-dried (105°C in oven, overnight), pre-weighed conical bottom glass centrifuge tube and centrifuged at 6000 rpm for 10 min. The harvested biomass was washed two times with sterilized deionized water and the centrifugation process was repeated. Then washed biomass was dried at 105 °C until getting the constant weight. After drying, it was allowed to cool in a desiccator and the final weight was recorded using an analytical balance (S234, Denver Instrument, Bohemia, NY). The loss of weight was calculated as grams of dry weight per litre. The biomass estimation was performed in triplicate and all values are represented as mean ± SD of three replications.

Identification of biomass producing yeast strain

The selected isolate was identified by genome sequencing. Universal fungal primers ITS1 (Forward 5' TCC GTA GGT GAA CCT GCG G 3) and ITS4 (Reverse- 5' TCC TCC GCT TAT TGA TAT GC 3')[15] were used for PCR amplification of the ITS 1, 2 and 5.8 S regions of the nuclear ribosomal gene complex. The amplification reaction was performed by using a DNA thermal cycler (BIO-RAD, Model -T100™ Thermal Cycler). The sequencing was performed according to the manufacturer's protocol using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems 3730XL) and deposited in GenBank. The obtained nucleotide sequence was used for similarity search and phylogenetic tree was constructed using MEGA7 (Molecular Evolutionary Genetics Analysis)[16] version 7.

Optimization of ultrasound assisted hydrolysate preparation

Ultrasound assisted hydrolysate preparation was carried out using a probe system sonicator (Lark Innovative Fine Teknowledge, Chennai, India) combined with a transducer and a metallic probe of 2mm. The hydrolysate preparation parameters such as ultrasound power density (UPD) (0.2 to 0.5 WmL^{-1}) and treatment time (5 to 25 min) were optimized by RSM using central composite design (CCD). The experimental design includes A 13 experimental runs were designed and each run carried out by taking 150 mL of sample in a 250 mL stainless steel beaker and dipped the probe in the sample upto 1 cm depth. During sonication, the desired temperature was controlled by placing the beaker in a water bath[17]. Each experiment was carried out with replicates and the dry cell biomass was considered as response. The obtained data were used for regression analysis and expressed an empirical model as follows, which was related to the response measured for the independent variables in this experiment.

$$Y = \beta o + \Sigma \beta i Xi + \Sigma \beta i Xi^2 + \Sigma \beta ij XiXj$$

Where Y is the response of biomass, βo, βi, βij is the intercept, the linear coefficient and the interaction coefficient respectively. The statistical significance and analysis of variance (ANOVA) was done, and plotted the three dimensional response surface curves to study the interaction among these factors.

Inoculum preparation

Inoculum was prepared by transferring a loop full of selected yeast strain from YPD (Peptone 20 gL^{-1}, yeast extract 10 g L^{-1}, dextrose 20 gL^{-1}, Agar 20gL^{-1}) agar slant into 50 mL of YPD broth at aseptic condition. Then the inoculated broth was maintained under aerobic conditions at 30 ^{o}C with agitation speed at 120 rpm in an orbital shaking incubator (REMI laboratory Instruments, India) for 48 h. The pH of medium was adjusted at 6.0 with 1N HCl/NaOH. For further yeast cultivation process, 10% v/v of newly prepared inoculum was used as seed inoculum. All these media were autoclaved before use at 121 ^{o}C for 20 min with 15 psi.

Cultivation of selected yeast strain in hydrolysate of *Chara*

The selected isolate was cultivated in 250 mL Erlenmeyer flasks contained 100 mL of hydrolysate. Prior to inoculate, the hydrolysate pH was adjusted to 7.0 using 1N NaOH or HCl and autoclaved at 121 ^{o}C for 15 min with 15 psi. Then, 10% v/v seed inoculum was added and incubated in a thermo controlled orbital shaker incubator at 27 °C for 72 h under constant shaking at 120 rpm. End of the experiment the final biomass and biomass productivity were evaluated. YPD broth was used as a control.

Antibacterial activity test

Antibacterial activity of yeast isolate against bacterium *Bacillus cereus* was done according to Roostita et al[18]. Nutrient agar (NA) plates were aseptically prepared for *B. cereus* by pour plate technique. Then freshly prepared yeast colony was carefully picked up from SDA medium and placed in the middle of NA plates with B. cereus. This was incubated at 35 ^{o}C for 24 h and measured the inhibition zone.

Analysis

The total reducing sugar concentration of hydrolysate was estimated by Dinitrosalicylic (DNS) acid method[19]. The protein concentration of yeast strain biomass produced from hydrolysate was estimated by Lowry et al[20].

Statistical analysis

All the experimental results were presented by the average value of triplicates with standard deviation (SD). The ultrasonic pretreatment parameters were statistically optimized at $p \leq 0.05$ confidence level using RSM and the data were further analyzed using one-way analysis of variance (ANOVA) using MINITAB 15software.

RESULTS AND DISCUSSION

Isolation and screening the potential yeast strain

Yeasts are widely distributed in nature. There are many reports in literature suggesting the ability of yeasts for various products production. However, there is an immense need to explore natural habitats to isolate potential yeast strain for enhanced production of products. Hence, the yeast isolation and screening is an important stage for evaluating the potential biomass producer from different sources. In this study, seven different yeast strains were isolated from soil sample collected from sugarcane bagasse dumping sites and designated as MMS1 - MMS7. All the isolates were stored at 4 ^{o}C in YPD slants. Batch cultures were prepared with 100 mL of crude extract of *Chara* sp for screening the potential yeast strain for biomass production. Among the seven isolates, only the strain MMS7 was selected after being cultivated on crude extract obtained from *Chara* sp biomass, where it gives the maximum biomass production of 12.44 ±0.11 g dwL^{-1} and biomass productivity was 0.1728 $gL^{-1}h^{-1}$ (Table 1).

Table 1 List of isolated yeast strains and screening for biomass production in crude extract of *Chara* sp. biomass

Culture Code	Final Biomass (g dw L^{-1})	Biomass Productivity (g $L^{-1}h^{-1}$)
MMS 1	10.46±0.12	0.1453
MMS 2	11.26±0.10	0.1564
MMS 3	10.21±0.11	0.1418
MMS 4	11.32±0.09	0.1572
MMS 5	11.21±0.07	0.1560
MMS 6	11.02±0.05	0.1530
MMS 7	12.44±0.11	0.1728

Identification of Yeast Strain MMS7

Sequencing the PCR amplified ITS regions of rRNA gene has gained the success of species identification in yeast *Candida*[15]. In the present study, 5.8S rRNA gene, ITS 2 and large subunit rRNA gene of selected yeast strain MMS7 were amplified by PCR and then sequenced by sanger dideoxy sequencing. The PCR amplified sequence was ~397 bp. Then the sequence similarity search was done by comparing the sequence of the selected yeast strain with the other yeast strains sequences, which are in the GenBank Database. The distance matrix was generated with length of 1000 replicates suing Jukes-Cantor method[21]. Then the evolutionary analysis was conducted by constructing the phylogenetic tree with 30 nucleotide sequences using the software MEGA7[16], after elimination of the gaps and missing data. The constructed phyolgenetic tree showed that the selected yeast strain MMS7 was nearly 100% close to the yeast strain *Cyberlindnera jadinii* PMM10-1793L (accession number: KP132001) (Figure 1). The phylogenetic analysis information revealed that the isolate MMS7 was corresponding to *Cyberlindnera jadinii* (anamorph: *Candida utilis*). Hence, this strain sequence was submitted to GenBank as *Cyberlindnera jadinii* strain MMS7, and the assigned GenBank accession number was MK942589.

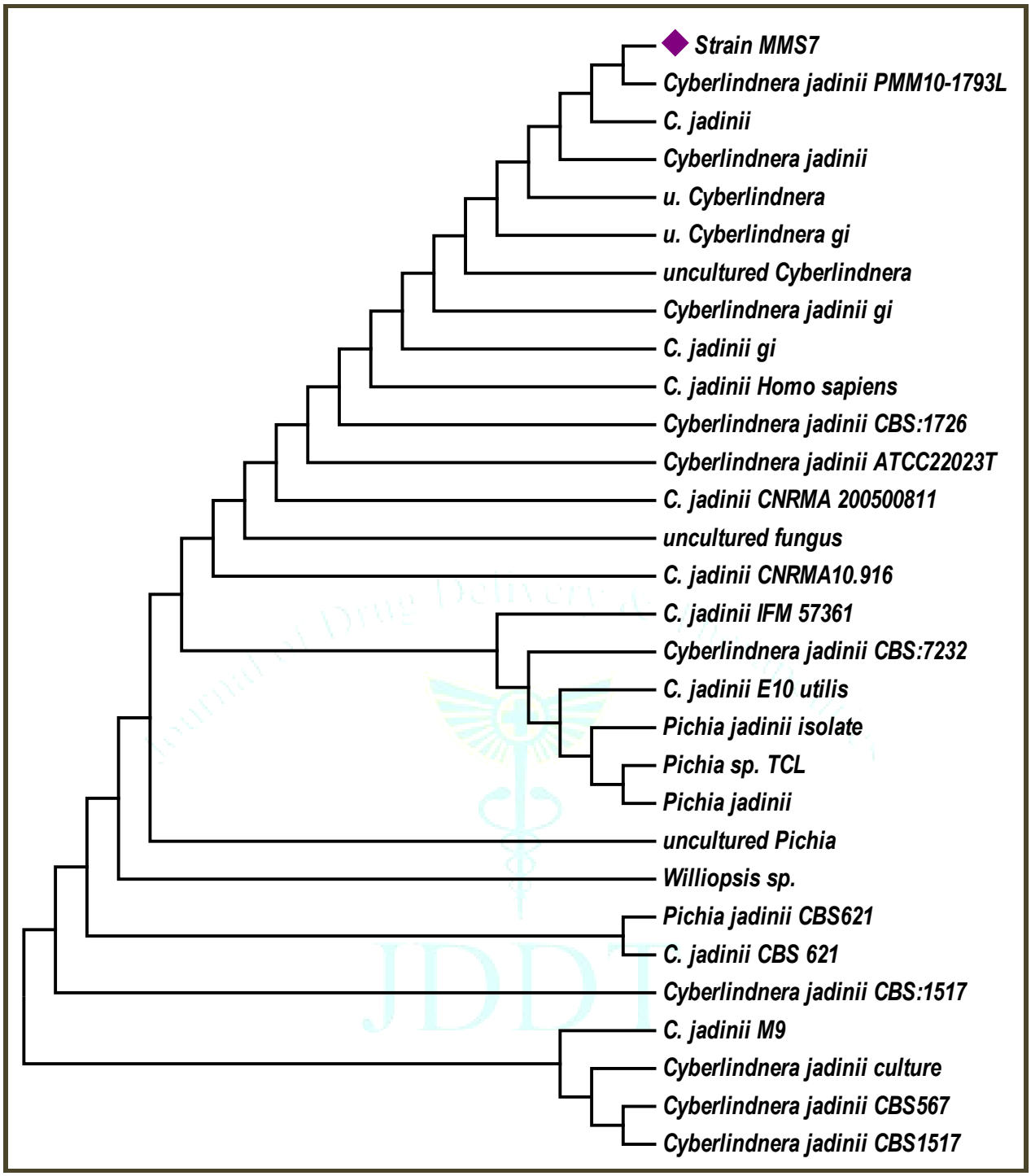

Fig. 1 Phylogenetic tree of isolate MMS7 (marked) is corresponding to *Cyberlindnera jadinii* PMM10-1793L

Optimization of ultrasonic pretreatment for hydrolysate preparation from *Chara* sp. and utilization for *C. jadinii* MMS7 cultivation

In the present study, the ultrasonic pretreatment process optimization for hydrolysate preparation from crude extract was studied using CCD of RSM. In order to find the optimum level of ultrasound power density and treatment time for this study, the selected yeast strain *C .jadinii* MMS7 biomass was considered as an indicator for these parameters optimization. A 13 experiments was designed for these two independent factors and all the experiments were carried out in duplicate. Table 2, showed the design matrix included two variables and the predicted and experimental values of response (Biomass production) of *C .jadinii* MMS7. The CCD analysis was done using coded units. The model was expressed by the following linear regression equation (Table 3) for the yeast biomass (Y) as a function of the effect of ultrasonic power density (X_1) and treatment time (X_2) on the *Chara* biomass for hydrolysate preparation by ultrasonic pretreatment.

$$Y_{CODED} = 15.022 - 0.1509X_1 - 0.4094X_2 - 0.9479X_1^2 - 0.9329X_2^2 + 0.5525X_1X_2$$

where Y is the biomass production (gL^{-1}), X_1 and X_2 are the coded value of ultrasound power density and treatment time time respectively.

The RSM results were plotted as the three dimensional response surface curves to find out the optimum level of independent variables for crude extract pretreatment to prepare hydrolysate and make it as a suitable medium for production of maximum biomass in *C .jadinii* MMS7. From the surface plot (Fig. 2), it was found that the ultrasound power density of 0.35 WmL^{-1} and treatment time 15 min as the optimum conditions for converting crude extract into hydrolysate by ultrasonic pretreatment, because at these conditions the yeast strain *C .jadinii* MMS7 gave the maximum biomass of 15.03 ± 0.04 gL^{-1}. This may be because of the hydrolysate with high dissolved nutrient contents, which were liberated from *Chara* biomass during the ultrasonic pretreatment at these conditions.

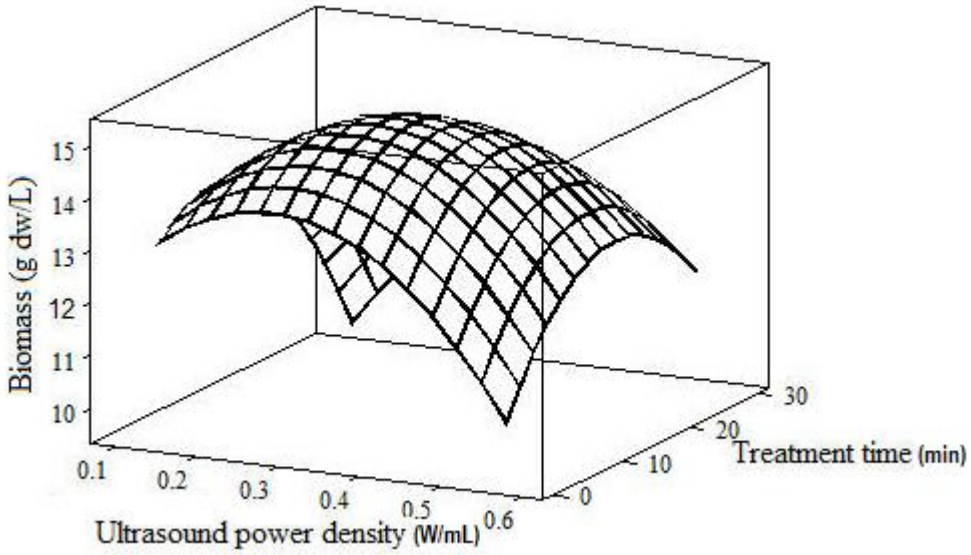

Figure 2. Response surface plot for biomass production of *C. jadinii* MMS7 cultivated in *Chara* sp. hydrolysate prepared by ultrasonic pretreatment

ANOVA was performed to examine the significance and adequacy of second-order polynomial equation. The ANVOA results were given in the Table 4. The coefficient of determination (R^2) value of 0.996 was closer to 1. Therefore, the correlation is better between the experimental and predicted values by the second order polynomial model[22]. Similarly, adjusted R^2 value of 0.993 was also very close to R^2 value. Hence, the model is well fitted to represent the effect of variables (ultrasound power density and treatment time) on ultrasonic pretreatment using RSM.

The small P-values indicate the higher significance of the corresponding variable. Therefore, the model obtained for this study was significant ($P \leq 0.05$) and suggesting that the ultrasonic pretreatment independent variables power density and treatment time could play a synergistic role on liberating the nutrient contents from the *Chara* sp. biomass. The statistical results of this study demonstrated that the ultrasonic pretreatment is a significant factor in prior utilization of crude extract of *Chara* sp.for production of biomass from novel yeast strain *C .jadinii* MMS7.

Table 2: Design matrix of the CCD for two variables with experimental and predicted values of *C. jadinii* MMS7 biomass

Run Order	Ultrasound power density (WmL^{-1})	Treatment time (min.)	Final Biomass ($g\ dwL^{-1}$)	
			Experimental	Predicted
1	0.200000	5.0000	14.12	14.2540
2	0.500000	5.0000	12.85	12.8473
3	0.200000	25.0000	12.24	12.3302
4	0.500000	25.0000	13.18	13.1335
5	0.137868	15.0000	13.48	13.3396
6	0.562132	15.0000	12.86	12.9129
7	0.350000	0.8579	13.81	13.7353
8	0.350000	29.1421	12.59	12.5772
9	0.350000	15.0000	15.03	15.0220
10	0.350000	15.0000	15.03	15.0220
11	0.350000	15.0000	15.00	15.0220
12	0.350000	15.0000	15.03	15.0220
13	0.350000	15.0000	15.02	15.0220

Table 3: Estimated regression coefficients of second order polynomial model for biomass obtained from *Chara* sp. hydrolysate using *C. jadinii* MMS7

Variables	Estimated Coefficients	t-value	*p*-value
Model	15.022	371.554	<0.001*
X_1	-0.1509	-4.720	<0.002*
X_2	-0.4094	-12.809	<0.001
X_1^2	-0.9479	-27.654	<0.001*
X_2^2	-0.9329	-27.216	<0.001*
X_1X_2	0.5525	12.223	<0.001*

R-Sq = 99.6% R-Sq(adj) = 99.3% * Significant

Table 4: Analysis of variance (ANOVA) for biomass obtained from *Chara* sp. hydrolysate using *C. jadinii* MMS7

Source	Degree of freedom (DF)	Sum of Squares (SS)	Mean Square (MS)	F-value	*p*-value
Regression	5	13.6287	2.72574	333.51	<0.001
Linear	2	1.5230	0.76152	93.17	<0.001
Square	2	10.8847	5.44233	665.89	<0.001
Interaction	1	1.2210	1.22102	149.40	<0.001
Residual Error	7	0.0572	0.00817		
Lack of fit	3	0.0565	0.01884	110.85	<0.001
Pure Error	4	0.0007	0.00017		
Total	12	13.6859			

Experimental verification

The crude extract of *Chara* sp. biomass was used for ultrasonic pretreatment at 0.35 WmL^{-1} for 15 min and prepared the hydrolysate. Then 100 mL of hydrolysate was taken in 250 mL Erlenmeyer flask as sole culture medium and inoculated *C. jadinii* MMS7 (10% v/v). The cultures were incubated at 27 °C with pH 7.0 for 72 h under constant shaking at 120 rpm. At the end of the experiment, the biomass was used for estimation. The maximum amount of biomass production using hydrolysate was prepared by ultrasonic pretreatment at 0.35 WmL^{-1} for 15 min, which was found to be 15.05±0.12 g dwL^{-1}, and also very close to the biomass concentration obtained in the experiment run 9 to 13 (Table 2). The maximum total reducing sugar concentration was observed as 83.21±0.03%. This study proves RSM as an adequate approach for optimization of pretreatment of *Chara* biomass for hydrolysate preparation as well as the studied hydrolysate proved to be better medium for maximize the biomass production by novel yeast *C. jadinii* MMS7 as compared with YPD broth (10.34±0.12 g dwL^{-1}).

Protein Estimation

The protein concentration of obtained biomass of *C .jadinii* MMS7 was estimated. Nalage et al[23] reported that the protein concentration of yeasts range from 47 to 53%, whereas the novel yeast strain *C. jadinii* MMS7 presents a total of 59.21±0.12%. Hence, this high protein concentration made by *C. jadinii* MMS7, is a remarkable protein producer utilizing *Chara* sp. hydrolysate as sole medium. This result indicates that the hydrolysate prepared from biomass of *Chara* sp. by ultrasonic pretreatment could be the best low cost medium for protein rich biomass production from *C .jadinii* MMS7.

Antibacterial activity test

In this study, a large inhibition zone of 24 mm was observed in NA plate. The yeast strain *C .jadinii* MMS7 isolated from sugarcane bagasse dumping sites have high antibacterial activity against *B. cereus*. This findings confirming the studies of Roostita et al.[18] and Younis et al[2], they tested various yeast species isolates isolated from various livestock products and milk and meat products on different pathogenic bacteria.

CONCLUSIONS

Large-scale production of protein rich yeast biomass could be easily improved by searching for new isolates from different sources. In the present study, a novel yeast strain *C .jadinii* MMS7 was isolated from soil sample collected from sugarcane bagasse dumping sites, and it is considered safe to be used in biotechnological applications. The ultrasonic pretreatment at 0.35WmL for 15 min was the optimum condition for hydrolysate preparation from *Chara* sp. biomass because hydrolysate prepared at these conditions, gave the maximum biomass of 15.05±0.12 gdw L^{-1} by *C .jadinii* MMS7. The protein concentration of *C .jadinii* MMS7 was remarkably high, therefore, this strain could be considered as promising single cell protein producing strain. Also, it has high antibacterial activity against to *B. cereus*. The exploitation of *Chara* sp. hydrolysate potential may have aggregate value for algae, being substrate for producing high amounts of yeast single cell proteins.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest to publish this paper.

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