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Isolation, Optimization and Extraction of Microbial Pigments from Marine Yeast *Rhodotorula Sp* (Amby109) As Food Colourants

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The use of synthetic dyes have been found to be unsafe to human health and there only some limited kinds of such dyes are permitted to be use. Hence there is an urgent need of natural food colorants to alternate the existing one. In general, the natural colors are quite common and rich in pigment producing microorganisms such as bacteria, yeast, fungi etc., among which the yeast are easily grown unicellular eukaryotes. In this present study, morphologically 30 different Marine yeast strains from 12 different samples collected from 6 different saltern areas of Kelambakkam, East Coast of Tamil Nadu, India and they were named as AMBY101 to AMBY130. Among the 30 strains 3 strains were capable of producing pigment. The pigments were extracted from all the three yeast strains using methanol as solvent and they were screened for antimicrobial activity against human pathogens such as *Escherichia coli*, *Enterococcus faecalis, Vibrio cholerae*, *V. parahaemolyticus*, *Salmonella* sp. and *Shigella* sp (obtained from AMET Microbial Culture Collection Centre). Based on the zone of inhibition, cell density and pigment production the marine yeast strain AMBY109 was potentially chosen for further study. The morphological and chemotaxonomic characteristics were confirmed that the potential marine yeast strain AMBY109 was belonging to *Rhodotorula Sp*. Response surface methodology (Box behnken design) were used to produce the pigment from the potential strain under different physicochemical parameters. Based on box behnken design and analysis of variance, the optimum culture conditions were found as: pH (8.54), temperature (34.29^{0}C), salinity (20.15ppt) and incubation period (48.24 hrs). With the optimum condition pigment production was predicted 0.7263 by this model and pigment production was observed 0.7152. From the results the study has suggested to use these readily absorbable natural pigments from *Rhodotorula* Sp. for an alternative of synthetic colorants in food industry.

Keywords: Pigments, Marine Yeast, *Rhodotorula* sp., Food colorants.

A well textured food, rich in nutrients and flavor, cannot be eaten unless it has the right color. Colors are one of the main visual properties of food and coloring of foods has been traditional practice for years. This practice has lead to the invention of synthetic colorants which have physical properties such as good stability and coloring ability (Pattnaik *et al*., 1997). Currently the commercial market is ruled by the synthetic pigments. Among the permitted synthetic pigments, some of them are toxic, carcinogenic and cause severe damage to vital organs (Duran *et al*., 2002). Nowadays, there is a growing interest on natural pigments due to their natural character, medicinal properties and nutritive value. Most of the natural pigments are extracted from plants like

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annatto, grapes, paprika, etc. and microorganisms like *Monascus, Rhodotorula, Bacillus, Achromobacter, Yarrowia, Phaffia* etc (Aberoumand, 2011 and Ahmad, 2012). Pigments like carotenoids, anthraquinone and chlorophyll have been produced from yeast, fungi, bacteria and algae and natural pigments possess anticancer activity, contain pro-vitamin A and have some desirable properties like stability to light, heat and pH (Joshi *et al*., 2003). Thus, natural colours in addition to being environment friendly, can also serve the dual need for visually appealing colors and probiotic health benefits in food products (Nagpal *et al*., 2011). Pigments come in a wide range of color among them some of which are water soluble (Tibor, 2007). Among the microbes, yeasts are traditionally used in food fermentation due to their less toxic in nature compared to other microbes. And yeast are distributed in almost every part of the aquatic environment including in marine and other hyper saline environments (Fell JW. 2001). Yeasts are unicellular microorganism a polyphyletic group of basidiomycetous and ascomycetous fungi with a unique characteristic of unicellular growth (Kutty & Philip 2008). Much marine yeast are good source of microbial pigments among them most important genera are *Metchnikowia, Kluyveromyces, Rhoaospoj-iaium, Candida, Cryptococcus, Rhodotorula* and *Toruiopsjs* (Hoog *et al.,* 2001). Compare than terrestrial yeast, marine yeast metabolites having more stable in different pH and temperature. Thus the present study was aimed to isolate a pigment producing marine yeast from different marine environment and apply these pigments as a food colourants in food industry.

MATERIALS AND METHODS

Isolation of the pigment producing Marine Yeast

The water and sediment samples were collected from different saltern area of Kelambakkam, East coast of Tamil Nadu, India. The collected samples were serially diluted up to 10^4. 0.1 ml of sample was taken from the dilution 10^2 to 10^4 and spreaded in Yeast Malt Medium. The plates were incubated at 28ºC for seven days. After the incubation period, the morphologically different pigmented colonies were subcultured in the respective agar plates and stored for further use (Chen *et al.,* 2009). And the isolated yeast were checked their salinity tolerance 0 to 5% Nacl added YM broth with the interval of 1%.

Extraction of pigment

The selected pigment producing yeast isolates inoculated in YM broth in rotary shaker incubated for 6 days at 28±2°C. And cells were harvested by centrifugation (3575g) for 15 mins the pellet was washed with sterile distilled water and spin for 15 min (894g) and weighed. The pellet was suspended with 5 ml of methanol and it was incubated in water bath at 60^0C for 15 min until all visible pigments were extracted and centrifuged (894g) for 15 min. The coloured supernatant was separated and filtered through Whatman no.1 filter paper and the coloured extracts were analyzed by scanning the absorbance in the wavelength region of 400-600 nm using the spectrophotometer. The total coloured content in the methanol extract was estimated by measuring the absorbance at *» max* (490nm) highest pigment producing isolates were selected for further analysis (Sasidharan *et al*, 2013)

Antimicrobial activity

The pigment was extracted from the potential marine yeast strain by using methanol as solvent and the antimicrobial activity of obtained pigment was checked against human pathogens such as, *Escherichia coli*, *Enterococcus faecalis*, *Vibrio cholerae*, *V. parahaemolyticus*, *Salmonella* sp., and *Shigella* sp (obtained from AMET Microbial Culture Collection Centre) in agar well diffusion method using methanol as control (Schillinger and Lucke 1989). After the incubation period the antimicrobial activity of pigment was determined by measuring the diameter of the zone of inhibition (ZOI) around the wells.

Identification of the potential pigment producing Marine Yeast

The maximum pigment producing potential strain was suspended in sterile water and a loopful of the suspension was streaked onto the YM agar plates and incubated at 28ºC for 6days. On the basis of standard morphological and chemo taxonomical tests the yeast strain was identified (Barnett, et al., 2000; Kurtzman and Fell 2006)

Statistical Optimization of potential Marine Yeast for pigment production by Response Surface Methodology

Response surface methodology (RSM) was used to investigate the increase the pigment

production from potential yeast. From the results obtained in RSM, Box behnken design was used to investigate the influence of four independent variables (Table 3) such as pH (7-10), salinity (0-35ppt), temperature (25-45^{0}C) and incubation period (24-72 hrs). (Karuppiah *et al*., 2013). The experimental data were fitted according to Eq. (1) as a second-order polynomial regression equation including individual and cross effect of each variable.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad ...(1)$$

Where, Y, β_0, β_i, β_{ii} and $^2_{ij}$ are the predicted response, a constant, a linear coefficient, a squared coefficient and an interaction coefficient, respectively. Eq. (1) was used to build surfaces for variables.

Statistical analysis

Multiple regression analysis, response surface plots and statistical analyses were performed using Minitab 15 Statistical Software® (Minitab Inc., PA, USA). In addition, analysis of variance (ANOVA) for the results was performed and a probability level of $p < 0.01$ was considered statistically significant.

Application of pigment as food colorants in water agar medium

The extracted 0.1 gm of pigment was mixed in water agar medium (100ml) and the color absorbance in the medium was visually observed in both agar plate and slant.

RESULT AND DISCUSSION

In general, marine microorganisms have potential applications. Among them, marine yeasts, provides a unique potential for the synthesis of functional biomolecules and stable in extreme conditions (Zaky *et al*., 2014). Moreover marine yeasts are able to produce many bioactive substances, such as amino acids, glucans, glutathione, toxins, enzymes, phytase and vitamins with potential application (Chi *et al*., 2009; Sarkar *et al*., 2010). In this study totally 30 different yeast strains were isolated from 6 different stations (Sediment and water samples) samples, collected from Kelambakkam saltpans, East Coast of Tamil Nadu, India and they were named as AMBY101 to AMBY130 (Table 1). All the isolated yeast strains had shown maximum growth in 4 to and 5% Nacl added YM broth. Fell, 1967 found living yeasts in the Indian Ocean from the surface down to a depth of 200 m. In his study highest population of yeasts were grouped according to their distribution. Ubiquitous species such as *Rhodotorula rubra* and *Candida atmospherica* in all water masses. Isolation of yeasts was done at a depth range of 200–1000 m along the continental slope sediments of Arabian sea and the Bay of Bengal and the predominant genera identified were *Candida*, *Rhodotorula, Cryptococcus, Debaryomyces*, *Pichia* and *Trichosporon* (Lachance *et al*., 1976). Prabhakaran and Gupta, 1991 studied yeasts from sediment samples of the Indian EEZ. They found *Candida* and *Rhodotorula* as the dominant group of all the species Kathiresan *et al*. in 2011 isolated 10 ethanol producing marine yeast strains from mangrove sediments of Vellar estuary, Parangipettai. Ghosh *et al*., 2011 isolated yeast strains from West Bengal, India and it was identified as *Candida famata, Candida ipomoeae, Candida succiphila, Rhodototula mucilaginosa, ,Debaryamyces hansensii, Kodamaea anthophila* and *Pichia lachancei*. Among the 30 marine yeast strains, 3 strains namely AMBY101, AMBY109 and AMBY130 showed maximum cell density.

So, these three pigment producing strains were inoculated in YM medium and the pigments were extracted by using methanol as solvent. Based on the cell density and pigment produced by the yeast AMBY109, it was potentially selected for further study. In addition, based on the physical appearance the orange color pigment

Table 1. Isolation of marine yeast from different sediment and water samples

Nature of the sample	Total no. of strains Isolated	Morphologically different strains	Potential yeast strains – Cell density and Pigment	Name of the strain
Sediment	33	21	2	AMBY101 to AMBY121
Water	21	9	1	AMBY122 to AMBY130

Table 2. Observed response and predicted values of pigment production

S. No	pH	Temperature (^{0}C)	Salinity (ppt)	IP (hrs)	Pigment	Predicted
1	-	0	-	0	0.163	0.164
2	0	0	+	+	0.308	0.301
3	0	0	+	-	0.305	0.299
4	0	-	0	-	0.299	0.293
5	0	-	0	+	0.413	0.407
6	0	+	0	+	0.234	0.231
7	+	0	0	-	0.334	0.338
8	+	0	+	0	0.385	0.376
9	-	+	0	0	0.28	0.281
10	0	0	0	0	0.723	0.716
11	0	0	-	+	0.06	0.077
12	+	0	-	0	0	-0.025
13	0	0	-	-	0.058	0.075
14	+	0	0	+	0.421	0.425
15	0	0	0	0	0.723	0.716
16	0	-	-	0	0.01	0.007
17	-	0	0	-	0.444	0.435
18	0	+	0	-	0.343	0.340
19	-	-	0	0	0.356	0.353
20	-	0	0	+	0.361	0.352
21	-	0	+	0	0.194	0.211
22	0	+	+	0	0.169	0.167
23	0	-	+	0	0.274	0.279
24	+	-	0	0	0.323	0.333
25	0	0	0	0	0.703	0.716
26	+	+	0	0	0.263	0.277
27	0	+	-	48	0	-0.008

Table 3. Model coefficients estimated by multiple linear regressions (significance of regression coefficients) for production of pigment from Marine Yeast AMET 109

Term	Coef	SE Coef	T	P
Constant	-7.96108	0.312389	-25.485	0.000
pH	1.13687	0.052695	21.575	0.000
Temp. (Celcius)	0.17432	0.006379	27.328	0.000
Salinity (ppt)	0.02444	0.003043	8.033	0.000
IP (Hrs)	0.02489	0.002344	10.620	0.000
pH*pH	-0.07446	0.002850	-26.123	0.000
Temp. (Celcius)*Temp. (Celcius)	-0.00238	0.000064	-37.038	0.000
Salinity (ppt)*Salinity (ppt)	-0.00120	0.000021	-57.269	0.000
IP (Hrs)*IP (Hrs)	-0.00028	0.000011	-25.032	0.000
pH*Temp. (Celcius)	0.00027	0.000494	0.540	0.599
pH*Salinity (ppt)	0.00337	0.000282	11.950	0.000
pH*IP (Hrs)	0.00118	0.000206	5.739	0.000
Temp. (Celcius)*Salinity (ppt)	-0.00014	0.000042	-3.207	0.008
Temp. (Celcius)*IP (Hrs)	-0.00023	0.000031	-7.528	0.000
Salinity (ppt)*IP (Hrs)	0.00000	0.000018	0.034	0.974

R-Sq = 99.74% R-Sq(pred) = 98.61% R-Sq(adj) = 99.44%

Table 4. Analysis of Variance for Pigment production from Marine Yeast AMET 109

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	1.01764	1.017639	0.072689	331.34	0.000
Linear	4	0.1633	0.194213	0.048553	221.32	0.000
Square	4	0.80094	0.800940	0.200235	912.75	0.000
Interaction	6	0.05331	0.053307	0.008884	40.50	0.000
Residual Error	12	0.00263	0.002632	0.000219		
Lack-of-Fit	10	0.00237	0.002366	0.000237	1.77	0.414
Pure Error	2	0.00027	0.000267	0.000133		
Total	26	1.02027				

produced by the marine yeast strain AMBY109 was found to be more colorful than other two strains respectively. Naghavi *et al.*, 2013 also observed the higher amount of carotenoid concentration (0.084±4.672 mg/g) and dry cell (0.367±3.5 g/L) weight using *Rhodotorula slooffiae* in YM medium. While checking the antibacterial activity of the pigments from three different marine yeast strains, the pigment extracted from *Rhodotorula* Sp. AMBY109 have showed strongest inhibition zone against all the pathogens compared to other two strains. Bowman (2007) also revealed that the pigment derived from some marine species having low and high molecular weight compounds with antimicrobial activity. The potential strain AMBY109 was identified based on morphological and chemo taxonomical characteristics. And it was confirmed that it was belonging to *Rhodotorula* Sp the pure culture of the strain was subcultured and stored for further use (Fig: 1).

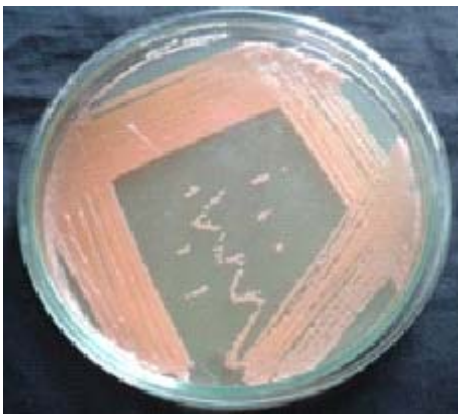

Fig. 1. Pure culture of the potential marine yeast strain *Rhodotorula* sp (AMBY109)

The use of statistical models to optimize culture medium components and conditions has increased in present-day biotechnology, due to its ready applicability and aptness. In the present study, box behnken design was employed to study the interactions among the four variables and also determine their optimal levels. It exploited in the present study enabled us to study and explore the culture conditions that would support a ~ 40% increased pigment production. A high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process for pigment production. The parameters of Eq. (1) were determined by multiple regression analysis by the application of RSM. The overall second-order polynomial regression equation showing the realistic relationship between pigment (Y) and four test variables in coded units was represented by Eq. (2) .

$$Y = -7.96108+1.13687+0.17432 +0.02444+0.02489-0.07446-0.00120-0.00028+0.00027+0.00337+0.00118-0.00014-0.00023+0.00000. \quad \text{Eq. (2)}$$

Based on the result obtained with the multiple regression analysis, individually it was having positive impact on pigment production. But it was observed that interaction of squared and some of interaction coefficient had a negative impact on pigment production (Table 4). The analysis of variance (ANOVA) by Fisher's statistical test result showed that the computed F value for linear regression was much greater than the tabulated (P) > F value. Therefore, the model terms temperature, pH, salinity and incubation period were found to be significant (Table 5). The goodness-of-fit of the model was checked by decisive the coefficient of determination (R^2) and adjusted R^2. When R^2 is larger then, the regression

has accounted for a large proportion of the total variability in the observed value of Y which favors the regression equation model (Haaland, 1990 and Mukherjee et al., 2009). The observed values of R^2 explain that the fitted model could explain 99.74% of the total variation and hence vouches for adequacy of the model. The adjusted R^2 value (99.44%) and Predication of R^2 (98.61) in the present study advocated a high significance of the model. These results reinforced that the response equation provided a suitable model for the box behnken experiment.

The interaction effects and optimal levels of the variables were determined by plotting the response contour plots. The response contour plots are shows the relative effect of all parameters on pigment production (Fig. 2). The lower and higher levels of all the factors did not result in

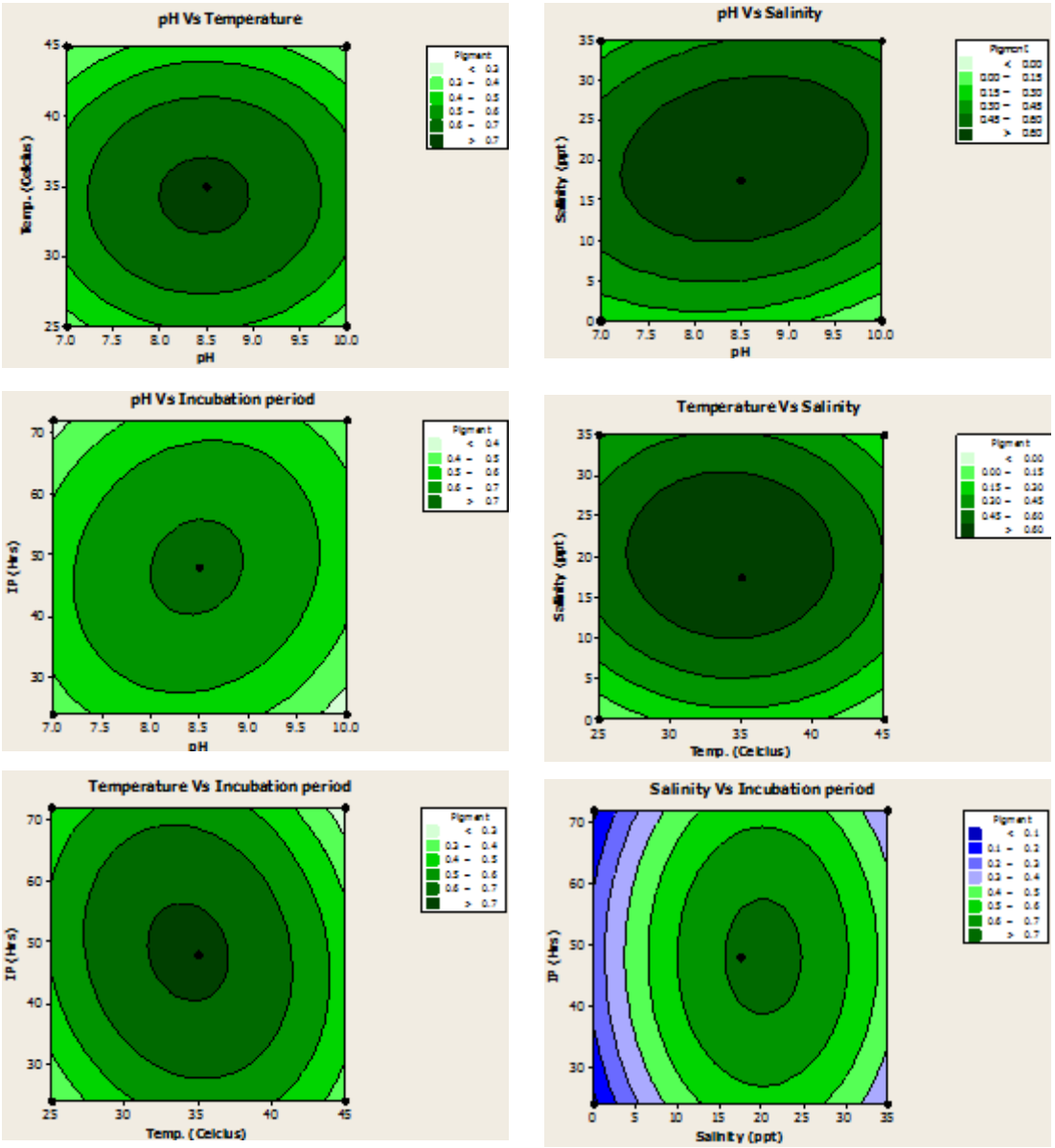

Fig. 2. Response contour plots of pigments production from potential marine yeast *Rhodotorula* Sp. (AMBY109) showing interactions between variables (temperature, pH, salinity and incubation period)

highest pigment production. The optimum conditions for maximum pigment production were proposed to be pH (8.54), temperature (34.29^{0}C), salinity (20.15ppt) and incubation period (48.24 hrs). The maximum pigment production 0.7263 was predicted by the model. The suggested medium composition was repeated. The validation experiment showed that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the model's authenticity. The potential marine yeast strain *Rhodotorula* sp AMBY109 has produced 0.7152 pigment under optimized culture conditions. Hamidid *et al*., 2014 have also reported that the production biomass was ranged from 0.04 to 0.84 g/l and the total carotenoid from 0.15 to 10.78 mg/l when optimizing *Halorubrum* sp under different parameters by using RSM. Recently, Tarangini and Mishra, 2014, also revealed that, the optimization of process parameters using RSM reported a 15% increase in the pigment yield than average yield obtained from the studied model by using the strain *B. safensis.* In fish processing industries some microbial colors are already in use fto enhance the pink color of farmed salmon (Venil *et al*, 2013).

The pigment obtained from the *Rhodotorula* Sp. strain was mixed with 100ml water agar medium in plates and test tubes and within 15 minutes the pigment color was absorbed. In recent times, some natural food colorants have a few commercial and acting as antioxidants (Dufosse, 2009). Currently there are some fermentative food grade pigments such as Monascus pigments, astaxanthin from Xantho-phyllomyces dendrorhous, Arpink Red from *Penicillium oxalicum*, riboflavin from Ashbyagossypii and carotene from *Blakeslea trispora* are commercially used in the markets and they which are considered as safe and approved by FDA : (European Commission, 2000). The rising demand by the consumers to replace the synthetic colors on food has pave way for production and high market value for the natural colorants. Moreover, the successful marketing of pigments derived from microbes in food and pharmaceutical industry is depending upon its functional role in the product (Venil *et al*, 2013). The results of the study has highlighted about the absorbance of readily absorbable natural pigment from *Rhodotorula sp* AMBY109 and the work also suggested to use this pigment as a alternative for synthetic colorants in food industry.

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