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ORIGINAL PAPER

High production of bacteriorhodopsin from wild type *Halobacterium salinarum*

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Abstract Bacteriorhodopsin (bR) is a trans-membrane proton pump found in the purple membrane of *Halobacterium salinarum*. This protein has high photochemical and photoelectric conversion efficiency and thermal stability, allowing it to withstand high temperatures, high salinity, and nutritionally-limited environments. The ability of this protein to convert light energy into chemical energy has applications that are mainly therapeutic/diagnostic and research-oriented. There is increasing demand for bacteriorhodopsin production in different fields. The present study maximized bacteriorhodopsin production using *H. salinarum*. The physical parameters of illumination, agitation speed, temperature, and nitrogen source were studied using a fractional factorial design to determine the optimal levels of each. The most suitable nitrogen source was determined to be peptone from meat. The optimal temperature was 39 °C, agitation speed was 150 rpm, and light intensity was 6300 lux for bR production. Under these conditions, the maximum bR yield was 196 mg/l, which is about 4.23 fold greater than those obtained with basal medium. The proposed strategies could be used for bR production using this archaeobacterium; the results are the highest reported thus far from a batch culture of *H. salinarum*.

Keywords Bacteriorhodopsin · Fractional factorial design · *Halobacterium* · Purple membrane · Proton pump

Introduction

Bacteriorhodopsin (bR) is a trans-membrane protein found in the archaeobacteria, mainly *Halobacterium salinarum*, that function as a light-dependent proton pump (Soppa 2006). This protein is found in 2D the crystal lattice known as the purple membrane (PM), which is naturally present in the plasma membrane of archaeobacteria. Under growth conditions favorable for induction of PM biogenesis, PM patches can cover more than 50 % of the cell membrane (Henderson et al. 1990).

This protein strongly absorbs green light, pumps protons outward from the plasma membrane, and converts light into an electrochemical gradient. This gradient is in turn used for ATP production (Danon and Stoeckenius 1974; Koch and Oesterhelt 2005). The ability of bR to convert light energy into chemical energy has applications for optical appliances, electronic devices, chemical sensing, filter devices and for therapeutic/diagnostic applications and research (Puthenveetil and Vinogradova 2013; Kawasaki et al. 2001; Hampp and Oesterhelt 2004; Knoblauch et al. 2014). The therapeutic application of this protein includes treatment of degenerated retinal blindness and eye disorders, therapeutic vaccine therapy, treatment of malignant tumors and other diseases, gene transcription regulation, drug delivery, transport and release of drugs, cell signaling, inducing apoptosis or death of neoplastic cells, control of cell signaling, fabrication of neuro-stimulation devices, and pharmaceutical applications (Koch and Oesterhelt 2005; Grout 2000; Lanyi and Pohorille 2001; Patil et al. 2012; Dummer et al. 2011; Kahya et al. 2005).

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Halobacterium salinarum is the most extensively studied archaea for production of bR (Lee et al. 1998). This microorganism lives in high-light, salty, high-temperature environments. Most applications require large amounts of PM from this microorganism. This has motivated researchers to scale up culturing and biochemical preparation to develop cost effective methods of producing large volumes of bR, which is a key requirement of profitability (Hampp and Oesterhelt 2004). It is obvious that the optimization of media parameters plays an important role in increasing production of any metabolite in setting up of any microbial fermentation process.

Factorial design is an effective mathematical method to solve complicated multifactor and multilevel problems using only a few trials; it is widely used in biotechnological applications. These designs can identify the main effects for the factors studied and uncover existing interactions between parameters. Although this method can decrease the number of runs, the experimental results are still reasonably logical. Analysis of variance (ANOVA) and *t* test analysis are used to interpret the result of the designs to determine the optimal experimental condition (Mason et al. 2003; Box et al. 2005).

There are few examples of research on bR production by microorganisms. These studies take media components such as carbon and nitrogen sources into account to increase bR production (Shand and Betlach 1991; Young et al. 1997; Rogers and Morris 1978). The physical factors like temperature, light intensity and agitation speed also play significant roles in bR production by archeal strains. Few studies have focused on optimization of physical process variables along with nutrient constituents for bR production using valid statistical methods (Faezi Ghasemi et al. 2008).

The present study optimized the physical variables and nitrogen sources for bR production by *H. salinarum* DSM 671 using fractional factorial design (FFD) and one-factor-at-a-time, respectively. The interactive effects of all variables were also investigated. The Kolmogorov–Smirnov test was employed to test the validity of a normal distribution.

Materials and methods

Microorganism strain and culture preparation

Halobacterium salinarum R1 (DSM 671) containing no gas vacuoles was obtained from German Collection of Microorganisms and Cell Culture for production of bR.

The composition of the basal *Halobacterium* medium used in this study was as follows (g/l): yeast extracts 10.0; casamino acids 7.5; Na_3-citrate 3.0; NaCl 250; $MgSO_4 \cdot 7H_2O$ 20; KCl 2.0; $FeSO_4 \cdot 7H_2O$ 0.05; $MnSO_4 \cdot 7H_2O$ 0.2. The initial pH was adjusted to 7.2 by adding NaOH. These experiments were done in basal medium in 250 ml erlenmeyer flasks containing 100 ml of media.

Effect of nitrogen source on growth and bR production

These experiments were carried out in basal medium containing different nitrogen sources (7 g/l) (casein acid hydrolysate, bacteriological peptone, tryptone, meat extract, peptone from meat, peptone from casein). Yeast extract was used in all experiments at a concentration of 5 g/l. The flasks were incubated at 39 °C and 150 rpm. Cell growth and bR concentration were estimated in cell broth at 7 and 8 days.

Cell growth and bR concentration measurement

Cell growth was monitored over time by measuring optical density (OD) at 600 nm (OD_{600}) using a Jenway 6310 (Bibby Scientific, UK). Synthesis of PM was assayed during growth by measuring the absorbance at 560 nm. To measure the concentration of bR (Shand and Betlach 1991), first, 2 ml culture samples were centrifuged at 10,000 rpm for 15 min at 4 °C. The pellet was then resuspended in 1 ml of distilled water containing 30 µl DNase. Freshly prepared 4 M NaOH and NH_4OH were added at a ratio of 9:0.5:0.5 (v/v) to the sample in the dark and absorption was measured by spectrophotometer at 560 nm (A_{560}). The sample was bleached (retinal removal from purple membrane) by exposure to light for 48 h; the absorbance of the solution was then measured (A^{48}_{560}). The amount of bR was determined as the difference between bleached and unbleached absorption at 560 nm. Because the molecular weight of bR is 26 kDa and the molar extinction coefficient is 63,000/M cm, the bR concentration was calculated as:

$$\text{Bacteriorhodopsin}\left(\text{g}/\text{l}\right) = 26,000 \times \left(\text{A}^{\text{o}}_{560} - \text{A}^{48}_{560} \Big/ 63,000\right) \quad (1)$$

FFD for physical parameter optimization

To identify which fermentation condition had a significant effect on bR production, a FFD was developed using Minitab 15 (Mathworks, USA). The physical parameters of temperature (37–39 °C), agitation speed (110–150 rpm), and light (3150–6300 lux) were selected as the factors for bR production by *H. salinarum*. A 2^{3-1} FFD produced 20 sets of experiments, which were performed in duplicate. The coded levels for the factors are listed in Table 1. Each factor was examined at a high level (coded +1), central level (0), and a low level (coded −1).

Table 1 Coded levels (in parentheses) and real values for the experimental design and results of the 2^{3-1} fractional factorial design

Runs	*A*	*B*	*C*	Y5	OD5	Y7	OD7	Y8	OD8
1	37 (−1)	110 (−1)	6300 (+1)	33	0.97	82.5	1.596	66.8	1.809
2	39 (+1)	110 (−1)	0 (−1)	16.5	1.028	67.3	1.51	104.4	1.68
3	37 (−1)	150 (+1)	0 (−1)	31.4	1.76	52.8	2.07	36.7	2.193
4	39 (+1)	150 (+1)	6300 (+1)	72.2	1.886	130	2.5	163	2.751
5	37 (−1)	110 (−1)	6300 (+1)	10.4	0.97	78.8	1.596	54.8	1.809
6	39 (+1)	110 (−1)	0 (−1)	15	1.028	68.9	1.51	113.1	1.68
7	37 (−1)	150 (+1)	0 (−1)	38.8	1.76	41.7	2.1	43.7	2.193
8	39 (+1)	150 (+1)	6300 (+1)	90	1.886	136.2	2.5	154	2.751
9	37 (−1)	110 (−1)	6300 (+1)	39.6	1.06	83.4	1.53	67.3	1.788
10	39 (+1)	110 (−1)	0 (−1)	16.1	0.96	51.6	1.26	82.1	1.452
11	37 (−1)	150 (+1)	0 (−1)	36.7	1.62	47.5	2.1	45.4	2.154
12	39 (+1)	150 (+1)	6300 (+1)	92.4	2.04	131.6	2.62	187.4	2.793
13	37 (−1)	110 (−1)	6300 (+1)	38	1.06	79.2	1.53	62.7	1.788
14	39 (+1)	110 (−1)	0 (−1)	18.9	0.96	54.5	1.26	66.8	1.452
15	37 (−1)	150 (+1)	0 (−1)	43.7	1.62	53.2	2.073	40.3	2.154
16	39 (+1)	150 (+1)	6300 (+1)	101.9	2.04	120	2.62	144	2.793
17	38 (0)	110 (−1)	0 (−1)	19.8	1.042	36.7	1.65	92.4	1.85
18	38 (0)	130 (0)	3150 (0)	24.7	1.042	59.8	1.65	103.9	1.85
19	38 (0)	150 (+1)	6300 (+1)	25.17	1.24	76.3	1.67	133.7	2.009
20	38 (0)	150 (+1)	6300 (+1)	25.17	1.24	62.3	1.67	135.7	2.009

A is the coded level of temperature (°C), *B* is the coded level of agitation speed (rpm) and *C* is the coded level of light

Results are averages from three replicates

Statistical analysis

Empirical fitting of the experimental data was obtained by polynomial regression based on analysis of variance (ANOVA) using Minitab 15 (Mathworks, USA) (Zar 1994). The response monitored (*y*) was bR production in terms of OD. The statistical significance of the model equation was determined by the results of *t* tests and *p* values. Before ANOVA, the data were evaluated for normality (Kolmogorov–Smirnov test), homogeneity of variance, and linearity of the model.

Optimal cultivation time

The production medium was collected at 5, 7 and 8 days and the variation in OD and bR production was recorded. Cultivation times were compared using the student's *t* test to evaluate the statistical significance of the difference between sample means. The results were considered significant at $p < 0.05$.

Results and discussion

Initial culture and bR production capacity

Initial cultivation of *H. salinarum* was carried out at 37 °C and 110 rpm using the basal medium. The growth conditions and bR production are described as a function of OD_{600} and OD_{560}, respectively. The cell culture experiments were conducted over 12 days and samples were collected daily for analysis of cell growth and bR concentration. Figure 1 shows the growth curve, bR concentration, and bR to biomass ratio (mg) of *H. salinarum*. As shown, the *Halobacterium* reached maximum growth after 7 days (OD_{600} 2.95) and then entered a stationary phase. The bR concentration followed the same pattern and maximum

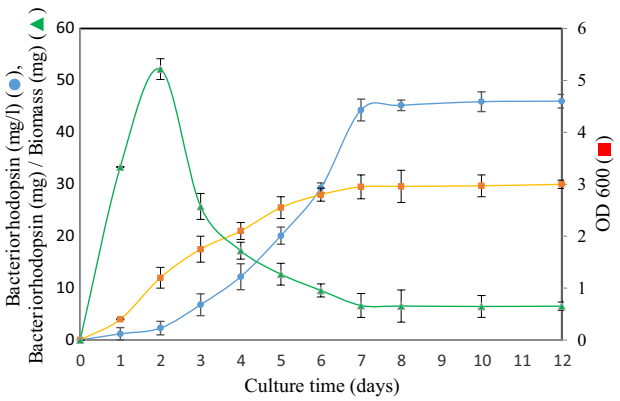

Fig. 1 Growth curve (*square*), purple membrane synthesis (*circle*) and bR (mg)/biomass (mg) ratios (*triangle*) of *H. salinarum*

basal production (46 mg/l) was observed at the end of the logarithmic phase (7 days). The results of analysis showed that bR production followed a pattern of increase, attained 46 mg/l at 7 days, and then remained constant. The bR production was, thus, measured at 5, 7 and 8 days.

The bR to biomass ratio (mg) increased up to 2 days after cultivation and then began to decrease in response to oxygen depletion in the culture medium; however, the concentration increased as the biomass increased. Studies suggest that oxygen plays an important role in regulating the biosynthesis of bR at the transcriptional and post-transcriptional levels. In fact, low concentrations of oxygen in the culture medium can provoke bR biosynthesis. This is in accordance with the findings of previous studies that quantified the mRNA level from the bacteriorhodopsin gene of wild-type *H. salinarum* under conditions in which oxygen levels were steadily depleted. The bacteriorhodopsin transcript levels increased in response to the steadily-decreasing oxygen level for the first 48 h (Beard et al. 1997; Müller and DasSarma 2005; Schmid et al. 2007; Shand and Betlach 1991).

Optimal temperature range

To determine the optimal temperature for *H. salinarum*, initial cultivation was carried out at 37, 39, 42, 45, and 47 °C and 110 rpm for 12 days in the basal media. Growth was described as a function of OD_{600}. Figure 2 indicates that all experiments showed the same growth pattern and that OD_{600} increased up to 7 days and reached a plateau afterward. It can be concluded from Fig. 2 that the highest rate of growth occurred in cultures incubated at 39 and 37 °C. The approximate optimal growth temperature that provided the highest growth rate for this strain was 37–39 °C. A noticeable adverse effect of high temperature was observed during testing; therefore, subsequent optimization steps were performed at 37, 38, and 39 °C.

All previous reports involving this organism were conducted at suboptimal growth temperatures. Some researchers have recommended that haloarchaeon should be grown at below optimal temperatures (37–40 °C). The motive for this recommendation was in part for convenience and out of concern for oxygen solubility at high salt contents; oxygen solubility could be an issue at near-maximum temperatures. High temperatures and extended incubation times can also cause rapid evaporation and dryness of the culture medium (Robinson et al. 2005).

Fractional factorial design

Because temperature, light intensity and agitation speed affect oxygen concentration, these physical factors significantly affect bR production. In this study, *H. salinarum* was cultivated on basal medium for 8 days at a light intensity of 0–3600 lux at different temperatures and agitation speeds (Table 1). Samples were collected at 5, 7, and 8 days for analysis. The results show the effect of these variables on bR production using FFD. The effect of each factor and their interactions were obtained at a confidence interval of 95 %. OD_5, OD_7, and OD_8 were recorded at 5, 7, and 8 days, respectively. The respective bR concentrations (mg/l) were measured and denoted as y_5, y_7, and y_8.

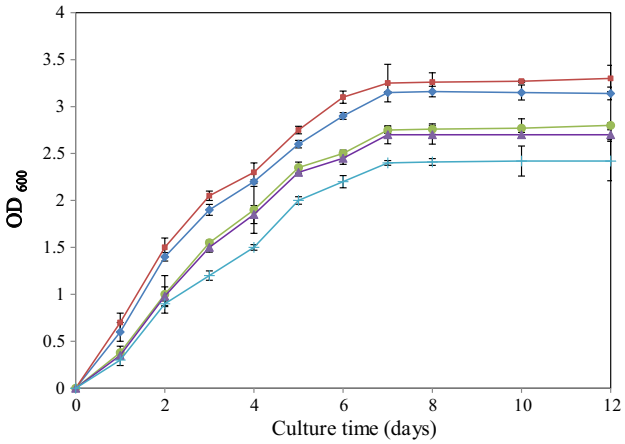

Fig. 2 Growth curve of *H. salinarum* incubated at 37 °C (*diamond*), 39 °C (*square*), 42 °C (*circle*), 45 °C (*triangle*), 47 °C (*plus symbol*)

Table 1 shows that bR production at 37 °C increased as the light intensity increased from 0 to 6300 lux and agitation speed increased from 110 to 150 rpm. This was also true on all days at the other temperatures (38 and 39 °C).

Table 1 shows that *H. salinarum* cultivated under high illumination (6300 lux) at high agitation speed (150 rpm) and high temperature (39 °C) attained the highest bR production levels of 101.9, 136.2, and 187.4 mg/l at 5, 7, and 8 days, respectively. The bR yield increased from 45.2 to 187.4 mg/l, which is 4.23 fold higher than for basal medium by simply varying the physical parameters of temperature, agitation speed and light intensity.

This finding is in accordance with those from previous studies which showed high levels of bR produced in response to high light density and limited oxygen. Under intensive light exposure, protons flux through the bR pumps and the phototropic growth of the microorganism will start (Sumper et al. 1976). Halophilic archaea thrive at high temperatures in saturated salt brine exposed to bright sunlight. Growth at 39 °C conforms, in part, to the high temperatures (37–50 °C) of the environments from which these organisms are isolated (Fendrihan et al. 2006). Faezi Ghasemi et al. (2008) showed that 150 rpm is the best agitation speed for bR production.

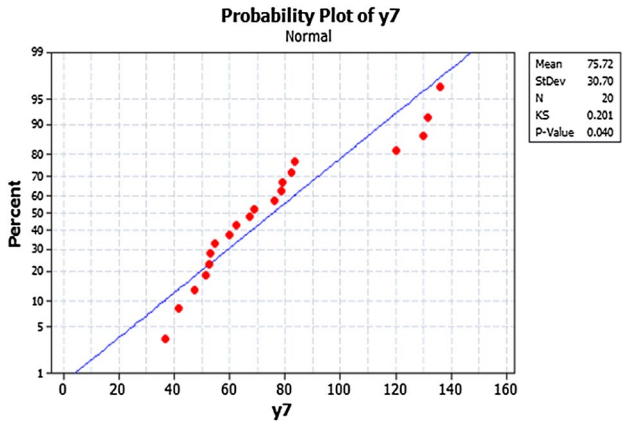

Fig. 3 The Kolmogorov–Smirnov tests for three variables

Kolmogorov–Smirnov tests used for data distribution analysis demonstrated that the data set was normally distributed. Figure 3 shows that the p values for the Kolmogorov–Smirnov tests confirmed the null hypothesis that the variables are normally distributed ($p \geq 0.05$).

ANOVA

The results at each point of the experimental design for bR production are listed in Table 1. Fitting of the data to the models and ANOVA showed that bR production was most suitably described by the following model:

$$y = 79.95 + 5.06A + 9.18B + 25.26C \quad (2)$$

This model predicts bR production by *H. salinarum* where y denotes bR production and A, B, and C are coded values for temperature (°C), agitation speed (rpm), and light intensity, respectively.

Positive values for the coefficients indicate a positive effect of that parameter on the reaction. All coefficients in this model had a positive value. Equation (2) indicates that the physical tested parameters have linear effects. The model was found to have an R^2 of 0.9697, indicating that the linear regression model is suitable for prediction and 96.97 % of the total variation resulted from the independent variables. Normally, models where $R^2 > 0.9$ have high correlations and fit the data (Haaland 1989); hence, R^2 in this regression model is relatively high and indicates good agreement between the predicted and experimental conversion. The predicted R^2 of 94.61 indicates that the actual versus predicted values for bR production are in good agreement. The adjusted R^2 was 0.9621. The similarity between R^2 and adjusted R^2 shows the adequacy of the model to predict the response.

Table 2 shows the results for ANOVA. The F value of 127.81 for the model indicates that it is significant. A value of $p < 0.05$ indicates that the model is significant and reliable. A value of $p < 0.001$ indicates there is only a 0.01 % chance that the model results from noise in the experiments; thus, it can be quantitatively assessed that the model represents the observations satisfactorily.

Temperature, agitation speed and light intensity were significant at $p < 0.05$, which indicate that these factors have desirable effects on bR production. The F value for lack of fit was 0.47, which indicates that it was not significant. As seen, bR production increased significantly as temperature, agitation speed and light intensity increased.

Optimization of cultivation time

The optimal culturing time was determined by comparing bR concentrations on different days using the paired student's t test. The average values and standard deviations of bR production at 5, 7, and 8 days are shown in Table 3. The average bR production at 5 days was 45.29 and the standard deviation (SD) was 28.30 days, which is significantly lower ($p < 0.01$, student's t test −8.148) than the bR production of 79.95 and SD 32.31 at 7 days.

The average bR concentration and SD at day 8 were higher than at day 7 (Table 3), but the difference was not significant ($p < 0.01$, student's t test −8.148). The lowest coefficient of variation was achieved at 7 days; thus, the optimum incubation time for the highest yield of bR was achieved at 7 days and cultivation for more than 7 days is not recommended.

Table 2 ANOVA for the model developed for bR production

Source	Sum of square	DF	Mean of square	F value	p value	Standard deviation	Coefficient
Model	15,188.1	3	5062.7	127.81	0.00021	1.573	79.95
Temperature	3630.1	1	3630.1	91.64	0.0047	1.573	15.06
Agitation speed	1346.9	1	1346.9	34.00	0.0032	1.573	9.18
Light intensity	10,211.1	1	10,211.1	357.79	0.0025	1.573	25.26
Residual	475.3	12	39.6				
Lack of fit	752	5	150.41	0.47	0.001		
Pure error	475.3	12	39.6				
Corr. total	15,663.4	15					

Table 3 Average values, standard deviation and coefficient of variation for bR production after 5, 7 and 8 days

Sample day	*p* value	Average	SD	SD mean	Coefficient of variation
Y5	0.0041	45.29	28.30	7.07	
Y7	0.0023	79.95	32.31	8.08	40.41
Y8	0.1	89.53	48.75	12.19	54.45

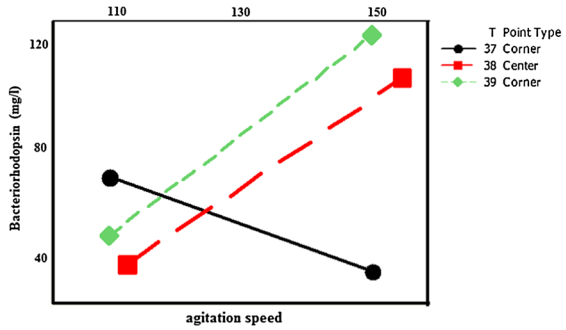

Fig. 4 The interactive effect of temperature and agitation speed on bR production

Mutual effect of process parameters

Instead of studying single variable the interactions are investigated, which are significant and important for a comprehensive optimization study. Figure 4 shows the interaction effects of temperature, agitation speed and light intensity on bR production. At 37 °C, an increase in agitation speed from 110 to 150 rpm decreased bR production, but at 38 and 39 °C, it increased production. Figure 5 shows that, at all temperatures, an increase in light intensity from 0 to 6300 rpm increased bR production. Figure 6 shows that, at a constant temperature at 130 rpm, an increase in light intensity from 0 to 6300 lux decreased bR production; but at 110 and 150 rpm, the increase in light intensity increased bR production.

These results are similar to those of Faezi Ghasemi et al. (2008), who used an agitation speed of 150 rpm for optimal production of bR, but who found that the effect of light intensity was greater than the effect of agitation speed. An increase in temperature substantially increased bR production. This is not surprising, since halobacteria live in extreme environments distinguished by hypersalinity, high solar radiation, and high temperature (Kennedy et al. 2001).

Effect of nitrogen source on growth and bR production

Halobacterium salinarum does not utilize simple nitrogen sources; for growth it relies on the complex nitrogen sources such as yeast extract and peptone (Lee et al. 1998).

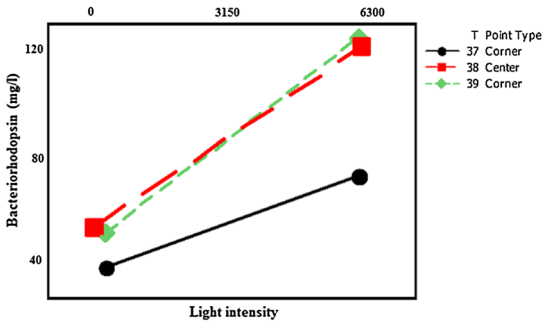

Fig. 5 The interactive effect of temperature and light intensity on bR production

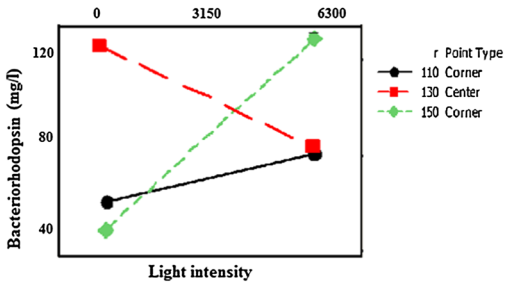

Fig. 6 The interactive effect of agitation speed and light intensity on bR production

It was found that the yield of bacteriorhodopsin production was low when yeast extract was used as the sole nitrogen source. When yeast extract was used in combination with other complex nitrogen sources, the growth yield increased. The preliminary results suggest that yeast extract has a complementary effect on promoting cell growth. This is in accordance with previous studies that used complex nitrogen sources in basal medium and supplemented it with other nitrogen components to improve productivity (Wang et al. 2008).

Casein acid hydrolysate, bacteriological peptone, tryptone, meat extract, peptone from meat, peptone from casein were tested as potential nitrogen sources for bR production at concentrations of 7 g/l for 7 and 8 days in basal medium at 6000 lux, 150 rpm and 39 °C. The yeast extract was used in all experiments at a constant concentration of 5 g/l. Figure 7 shows that the highest bR production was obtained using peptone from meat, bacterial peptone, and meat extract, respectively. This finding is consistent with those from Lee et al. (1998) and Faezi Ghasemi et al. (2008), who reported that *H. salinarum* growth relies on complex organic nitrogen sources such as yeast extract and peptone. It can be said that organic sources increased cell concentration and metabolite production over those of inorganic nitrogen sources. Essential amino acid(s) in some

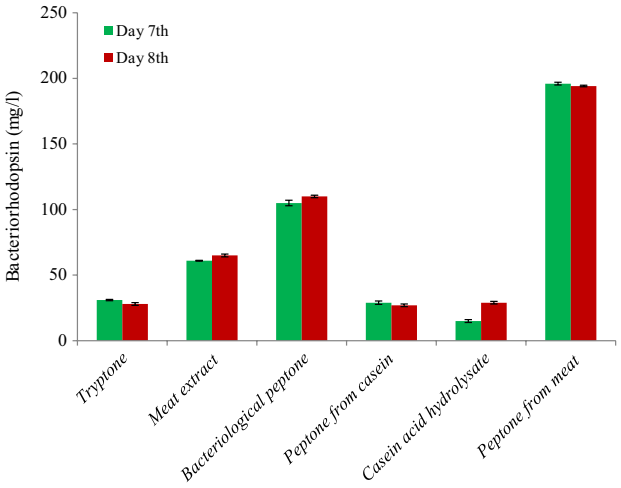

Fig. 7 Concentration of bR obtained with different nitrogen sources on days 7th and 8th

microorganisms cannot be synthesized from inorganic nitrogen sources using cultured cells (Wang et al. 2008). The highest bR production was about 196 mg/l using peptone from meat, which is about 4.3-fold higher than in basal medium.

Conclusion

The present study examined the effects of physical parameters and nitrogen sources on bR production by *H. salinarum* in shaker flasks using fractional factorial design. The results showed a significant increase in bR production simply by varying the physical parameters. The optimum physical condition for maximum production of bR was a temperature of 39 °C, an agitation speed of 150 rpm, and light intensity of 6300 lux; under these conditions, productivity of bR improved from 0.26 to 1.11 mg/l h. The bR yield increased from 45.2 to 196 mg/l at 7 days, about 4.33-fold higher than in basal medium by adjusting the physical parameters and nitrogen sources. This is higher than the results reported by Kahaki et al. (2014), who overproduced the bR gene in *E. coli* and obtained a bR concentration of 191 mg/l. Faezi Ghasemi et al. (2008) increased the bR concentration about 3.49 fold over the use of basal medium by optimization of all parameters in culture medium; this was also lower than the results of the present study.

Peptone from meat powder in a medium formulation increased production yield and appear to be a good ingredient that is simple to use in culture medium. Optimization of operating variables in bioreactor fermentation requires further investigation. The data obtained from batch culture can be used for development of fermentation processes for bR overproduction in the future. The methodology has proven that it is suitable for the design and optimization of bR production.

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