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Production of single cell oil by using cassava peel substrate from oleaginous yeast *Rhodotorula glutinis*

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ABSTRACT

Oleaginous yeasts seem to be the promising source for the production of lipid. In this context *Rhodotorula glutinis* was investigated utilizing an agro-industrial waste cassava peel. Medium optimization was performed by response surface methodology (RSM) with Box-Behnken design under submerged fermentation to improve the lipid yield. The results of RSM revealed that the highest lipid production of 58% lipid/dry biomass may be achieved when the culture was grown for 3 days at 30 °C under 120 rpm shaking, pH 5.45 in a medium containing 12% (w/v) cassava peel and 8.03% (v/v) yeast inoculum. Scanning electron microscopic studies confirmed complete colonization of starch granules by yeast. The process was further up scaled in a 7 L bioreactor and an yield increment of 9% was observed. Overall, the above results clearly displayed the potential of *R. glutinis* to utilize cassava peel waste for the single cell oil (SCO) production.

1. Introduction

Both, a global increase in population and the need for the transportation fuel has created a serious urge to find out the alternative fuel resources with specific focus on biomass which seems to be ideal feed stock to meet the demand of fuel (Sitepu et al., 2014; Coma et al., 2017). Most of the agro residues and agro-industrial wastes are dumped in landfills/roadside which contribute to green house gas emission. The valorisation of these residues to produce biofuel or single cell oil is a meaningful option (Didaskalou et al., 2017). Cassava peel generated during processing of tapioca starch may be a useful resource to be investigated for SCO production by using amylolytic oleaginous yeast. Production of lipid by employing oleaginous yeast has been studied for more than 80 years and researchers reported the variation in their fatty acid profiles with the production conditions despite of their similar taxonomic affiliations (Sitepu et al., 2013). Amylolytic microbes can utilize not only the starchy waste but also able to use broad range of agro-industrial wastes for the production of lipid (Amaral et al., 2012). Liu et al. (2015) utilized corn cob hydrolysate to produce 47% lipid content by *R. glutinis* CGMCC 2703. Similarly, lignocellulosic biomass hydrolysate proves to be good substrate for accumulation of lipid (34.5%) in *R. glutinis* BCRC 22360 (Yen and Chang, 2014). For an increase in microbial lipid production, optimization of nutritional factors and culture conditions during the process of fermentation are required. Environmental factors (temperature, pH), incubation time and the typical behaviour of the microorganism affect not only the percentage of lipid accumulation (Razaghi et al., 2016) but also the composition of fatty acid produced (Ageitos et al., 2011). The ratio of C16 or C18 species is also affected by carbon source and the culture medium for yeast cultivation (Karthikeyan et al., 2017). Along with classical approach of 'One variable at a time', response surface methodology (RSM) has also been used in optimizing medium and culture conditions (Awad et al., 2011) for biotechnological and industrial process (Saran et al., 2015). In our earlier studies, amylolytic yeast *R.glutinis* NRRL Y-1091 was found to be very promising candidate for lipid production (Chaturvedi et al., 2018a).

In present research investigation, the selected strain *R. glutinis* was investigated for production of lipid by using cassava peel using 'one variable at a time approach' and response surface methodology. RSM helped to select the most effective fermentation parameters to maximise lipid production from oleaginous yeast.

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2. Materials and methods

2.1. Materials

Dextrose, malt extract, peptone and yeast extract were purchased from SRL (Sisco Research Laboratories Pvt. Ltd., Mumbai, India), HiMedia Laboratories Pvt. Ltd. Mumbai, India and CONDA Spain. All other chemicals used were of analytical grade and high purity. Cassava peel was collected from local market, it was peeled, dried and grounded by milling.

2.2. Microorganism and culture conditions

Yeast strain *R. glutinis* NRRL Y-1091 was selected on the basis of earlier experimentation (Chaturvedi et al., 2018a). This strain was capable of utilizing different type of starch due to its capability to produce amylase enzyme.

2.2.1. Preparation of inoculum

The yeast inoculum was prepared by transferring loop full of culture from slant in to MGYP broth (pH 7.0) containing %: glucose, 2.5; malt extract, 0.3; peptone, 0.5; yeast extract, 0.3. Thereafter, the inoculated medium was incubated at 30 °C, 120 rpm for 5 days in an incubator shaker to obtain at yeast density of 10^9 cells/mL.

2.2.2. Experimental design for optimization of lipid production

Selected culture of *R. glutinis* was incubated for growth and lipid production under submerged fermentation (SmF). Real life starch waste (cassava peel) was investigated for oil production and substituted in yeast phosphate soluble starch (YPSs) medium (Kimura et al., 2004). This submerged production media contained cassava peel (8, 10, 12% w/v), potassium monophosphate (0.1% w/v) and magnesium sulphate (0.05% w/v). The pH of medium was adjusted at three levels (4.5, 5.0, 5.5) to ascertain the optimum pH for lipid production. The media was sterilized at 15 bps pressure for 15 min.

All the flasks (250 mL) containing 50 mL of media were inoculated with yeast inoculum (8, 10, 12% v/v) and pH (4.5, 5.0, 5.5). These inoculated flasks were incubated at 30 °C under shaking condition (120 rpm) for 3, 5 and 7 days. Samples were collected and centrifuged at 1180×*g* for 12 min.

2.3. Disruption of cell wall for lipid quantification

Biomass was macerated in liquid nitrogen with the help of mortar and pestle. The resultant biomass was than suspended in distilled water and sonicated under cold condition for 3 min, 40 pulses (5 times) using Ultrasonic homogenizer (model 3000, Biologics Inc. USA). The homogenized preparation was then added to 20 ml mixture of chloroform and methanol (2:1), shaken and was kept for half an hour (Poli et al., 2013).

2.4. Lipid extraction process

The crude extract after filtration was collected and diluted 0.2 times with distilled water. The diluted sample was vigorously mixed and was kept at rest for complete separation of the biphasic system. Without disturbing the interface, the upper phase was removed and 10 ml of methanol was added to the lower phase, containing extracted lipids part. The lower phase was extracted repeatedly and collected in a pre-weighed container. The container was then kept in incubator (Shaker Lab Therm, Kuhner, Switzerland) at 37–40 °C for complete evaporation of the solvent, and the amount was quantified by gravimetric method. The specific yield of lipid ($Y_{P/X}$) was represented as g of lipid/g biomass (Kilcawley et al., 2002).

2.5. Experimental design and statistical analysis

The initial selection of fermentation variables was done by 'one-variable-at-a-time' approach. For maximising the lipid production, media components were optimized by Box-Behnken design based Response surface methodology (RSM) with the help of experimental design module of Design Expert-7 (Stat-Ease Inc., Minneapolis, USA). The designed model was generated, and significance of the model was checked as per single response analysis. The influencing interactive effect of factors with their respective levels was constructed based on an appropriate response surface model for response and then extrapolation was done to find a set of functional conditions that optimized to a great extent of response. The range and the levels of the independent variables viz. carbon source, pH, inoculum size and days of incubation, selected for the current study has been given in Table 1. A total of 27 experimental runs were generated through Box-Behnken design including 3 replicates at the centre point to allow a better estimate of the experimental error. The experiments designed by the software for the optimization study is presented in Table 2 and Table 3 with the values of variables at different levels along with their respective experimental and predicted response. The quadratic model was generated based on the experimental results and the significance of the model was checked by ANOVA analysis. Furthermore, the predicted values of levels were validated by performing the experiment in triplicate.

2.6. Yeast cell sample preparation for Scanning Electron Microscopy

The growth of yeast on starchy peel waste during the fermentation was observed by Scanning Electron Microscopy (SEM) analysis. The sample was harvested on 0, 3 and 7 days of incubation and processed for gluteraldehyde based fixing and dehydration in a gradient of acetone. After gold coating, the samples were analyzed by SEM (ZEISS EVO Series Model EVO 50, Germany).

2.7. Scale-up studies in bio-reactor

Lipid and biomass production was scaled up in a 7 L stirred tank bioreactor with working volume of 5 L (Applikon, Schiedam, Netherlands) controlled by Applikonbio-controller, Bioconsole AD11025. Previously optimized growth conditions were adopted for scale-up studies in bioreactor. The bio-reactor was equipped with automatic control of temperature (30 °C), pH (5.45), agitation rate (200 rpm) and aeration rate (1.0 L/min). The pH of media was controlled by the automatic addition of either 5% (v/v) H_2SO_4 or 5% (w/v) NaOH. Medium was inoculated with 8% (v/v) inoculum raised in previously described MGYP medium. After 3.18 days of incubation sample was finally withdrawn, centrifuged at 10,000 g for 10 min at 4 °C and processed for lipid content and biomass estimation.

3. Results

In the present work, the impact of culture conditions of *R. glutinis* on the lipid production was studied. Cassava peel waste was used as carbon source and cultural conditions of fermentation as inoculum size, pH and incubation period were optimized by using statistical tool of Box-

Table 1
Experimental range and coded levels for Box–Behnken design based response surface methodology (RSM) for increasing the lipid production by *R. glutinis*.

Code	Process variable	Range and level		
		−1	0	+1
A	Carbon source(%, w/v)	8	10	12
B	Inoculum size (%, v/v)	8	10	12
C	pH	4.5	5.0	5.5
D	Incubation time(days)	3	5	7

Table 2
Box-behnken experimental design matrix with respective response for lipid production by *R. glutinis*.

S. No.	Carbon source (% w/v)	Inoculation size (% v/v)	pH	Incubation time (Days)	Lipid production (% lipid/dry biomass)	
					Actual Value	Predicted value
1	8	8	5	5	6.37	5.51
2	12	8	5	5	19.38	18.52
3	8	12	5	5	11.48	10.62
4	12	12	5	5	10.29	9.44
5	10	10	4.5	3	10.75	12.12
6	10	10	5.5	3	31.47	31.82
7	10	10	4.5	7	58.31	56.24
8	10	10	5.5	7	4.99	1.91
9	8	10	5	3	10.11	9.68
10	12	10	5	3	23.37	22.94
11	8	10	5	7	22.85	24.13
12	12	10	5	7	21.41	22.69
13	10	8	4.5	5	24.17	24.35
14	10	12	4.5	5	26.14	26.32
15	10	8	5.5	5	10.31	10.99
16	10	12	5.5	5	4.37	5.05
17	8	10	4.5	5	16.34	16.52
18	12	10	4.5	5	45.90	46.08
19	8	10	5.5	5	22.17	22.86
20	12	10	5.5	5	4.44	5.12
21	10	8	5	3	15.14	14.71
22	10	12	5	3	6.41	5.98
23	10	8	5	7	13.79	15.07
24	10	12	5	7	18.54	19.83
25	10	10	5	5	28.43	28.85
26	10	10	5	5	29.30	28.85
27	10	10	5	5	28.80	28.85

Behnken design based RSM analysis.

3.1. Evaluation of selected oleaginous yeast for lipid production using cassava peel waste as carbon source

R. glutinis was selected for optimization studies because of its high amylolytic activity as well as its capability to accumulate lipid 53.14% lipid/dry biomass at 30 °C incubation temperature and 120 rpm shaking, by using cassava peel wastes (Chaturvedi et al., 2018a). Accumulation of lipid in the oleaginous yeast cells have been well established fact with more than 30 C/N ratio. However, much research has not been carried out with real life substrate like cassava waste which displays close to ideal C/N ratio of 28.1 (Dasguptaab et al., 2017).

3.2. Optimization of medium components for lipid production from oleaginous yeast R. glutinis

The present work deals with the optimization of four most influential factors including carbon source (A), pH (B), Inoculation size (C) and Incubation time (D) on the lipid production from oleaginous yeast *R. glutinis* (Table 1). Table 2 displayed that all selected factors and related levels showed varied lipid production levels predicted to be in the range of 1.91–56.24% lipid/biomass. The predicted values generated by the software were also matched with the experimental values (4.99–58.31% lipid). The ANOVA analysis of results showed that quadratic model was statistically significant (p value < 0.0001) and lack of fit was insignificant (p value < 0.0653) (Table 3). The significance of model was also validated by 99.32% of coefficient of variation (R^2). The ANOVA table also revealed that among all the combinations, only one factor (pH) was insignificant (p value 0.6898) against the response of lipid production. All six-possible interactive among the factors were also significant which showed positive interactive effect of all selected factors. The interactive effect of factors on lipid production was also analysed by 3D contour plots against two experimental factors (Fig 1). Based on quadratic model of response, the equation in terms of coded variable and actual variables was as follows:

Lipid production (% lipid/dry biomass) = $+28.85 + 2.96 * A - 0.99 * B - 8.66 * C + 3.55 * D - 3.55 * A * B - 11.82 * A * C - 3.68 * A * D - 1.97 * B * C + 3.37 * B * D - 18.51 * C * D - 5.93 * A^2 - 11.89 * B^2 - 0.27 * C^2 - 3.05 * D^2$

The developed model was validated by performing the experiment under predicted optimal condition for lipid production by *R. glutinis*. The model proposed the optimal level of carbon predicted lipid production of 56.24% lipid/dry biomass. The experimental value of lipid production (58.31% lipid/dry biomass) under optimum condition was in close agreement with the predicted value. After Box-Behnken design based optimization, lipid production increased by 9% as compared to the result obtained under un-optimized conditions (53.14%).

3.3. SEM of lipid producing yeast R. glutinis

The fixed yeast cells along with starchy waste substrate were analysed by SEM. The growth and multiplication of cells were observed within 3 days of fermentation under different power of magnifications (Fig. S1). The starch granules were completely colonized by yeast cells.

3.4. Scaling up of the lipid production by R. glutinis

R. glutinis was cultured in a 7 L bio-reactor in order to evaluate the

Table 3
ANOVA based regression analysis for lipid production from *R. glutinis* for quadratic response surface model fitting.

Source	Sum of Squares	df	Mean Square	F Value	p-value	
Model	4153.85	14	296.70	125.25	<0.0001	Significant
A-Carbon source	104.81	1	104.81	44.24	<0.0001	
B-Inoculation size	11.83	1	11.83	4.99	0.0452	
C-pH	899.12	1	899.12	379.56	<0.0001	
D-Incubation time	151.34	1	151.34	63.89	<0.0001	
AB	50.38	1	50.38	21.27	0.0006	
AC	559.19	1	559.19	236.06	<0.0001	
AD	54.03	1	54.03	22.81	0.0005	
BC	15.60	1	15.60	6.59	0.0247	
BD	45.42	1	45.42	19.17	0.0009	
CD	1370.26	1	1370.26	578.45	<0.0001	
A2	187.52	1	187.52	79.16	<0.0001	
B2	754.58	1	754.58	318.54	<0.0001	
C2	0.40	1	0.40	0.17	0.6898	
D2	49.71	1	49.71	20.98	0.0006	
Residual	28.43	12	2.37			
Lack of Fit	28.04	10	2.80	14.71	0.0653	Not significant
Pure Error	0.38	2	0.19			
Cor Total	4182.28	26				

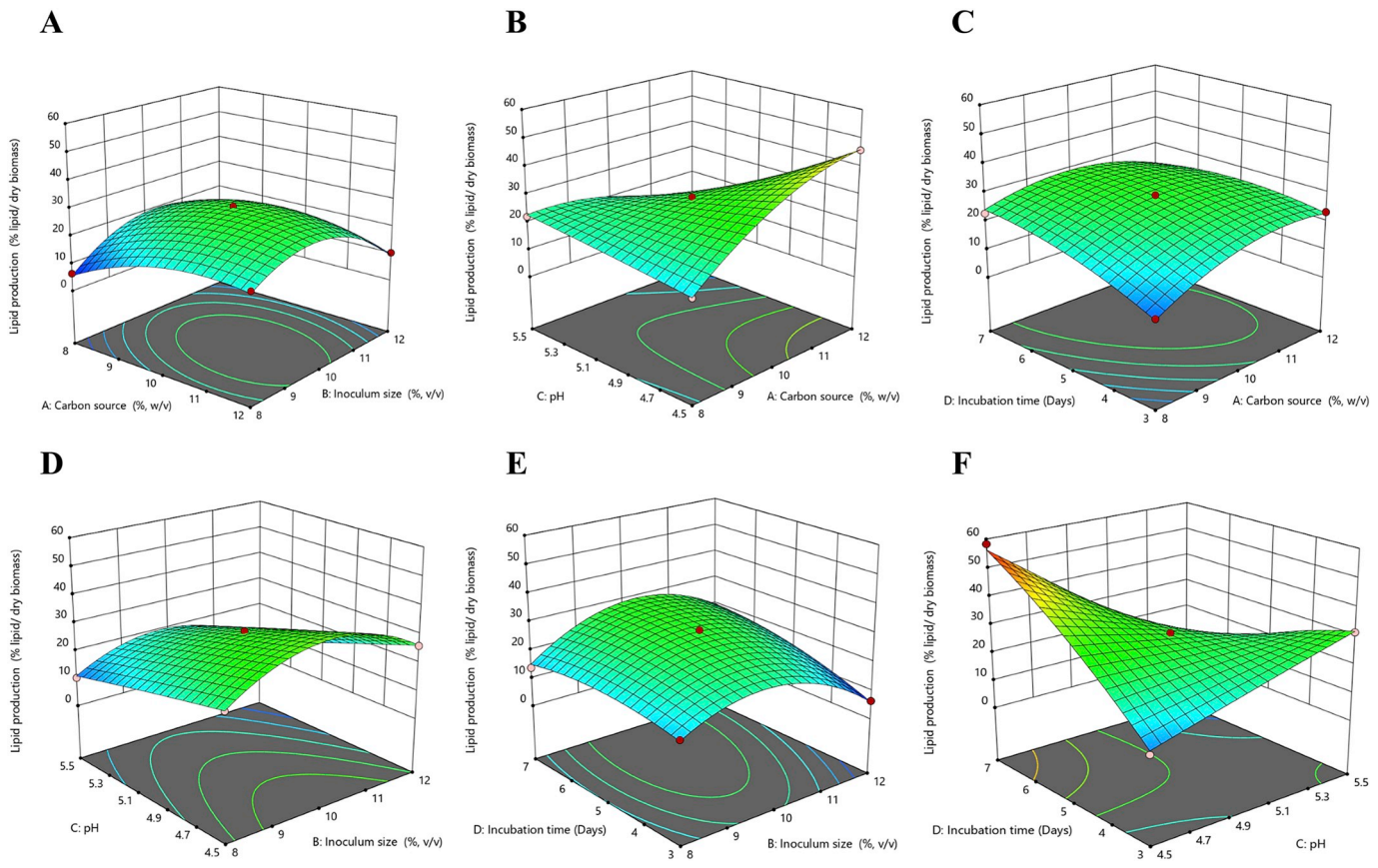

Fig. 1. Response surface 3D contour plots of Box-Behnken design for optimization of lipid production by *R. glutinis*. Figure shows the interaction between (A) Carbon source and Inoculation size; (B) Carbon source and pH; (C) Carbon source and Incubation time; (D) Inoculation size and pH; (E) Inoculation size and Incubation time; (F) pH and Incubation time.

lipid and biomass production on large scale in optimized medium containing 120 g/L cassava peel waste starch. At the end of the fermentation lipids was extracted and was 58.31% of dry biomass. Hence, around 9% increase in lipid content was observed after scale-up.

4. Discussion

The response surface methodology (RSM) approach involving a Box-Behnken design was adopted to optimize the media component for maximize lipid production by *R. glutinis*. In past several researches have reported lipid production from *R. glutinis* (Chaturvedi et al., 2018(a, b); Taskin et al., 2016; Magdouli et al., 2016; Liu et al., 2015; Yen and Chang, 2014). But cassava peel has not been investigated yet as a potential substrate. Therefore, in this study an attempt was made to produce lipid by using cheap carbon source such as agro-industrial waste to reduce the cost of biodiesel. In past, other researchers have also investigated other oleaginous yeast for production of biodiesel by using pulp and paper waste (Patel et al., 2017; Vyas and Chhabra, 2017). The elemental composition of cassava waste revealed that it contained carbon (38.73%), nitrogen (1.38%), potassium (8.21 ppm) and 28.1 C/N ratio, which was found to be quite suitable for yeast cells growth and lipid production. To enhance lipid and cell mass production, the optimization of key factors for maximization of oil yield is necessary (Wiebe et al., 2012). The oleaginous yeast like *R. glutinis* and *Candida utilis* were able to produce higher lipid/glucose yields (16%) under high glucose supply (Amaretti et al., 2010). In present study, 12% (w/v) of cassava starchy waste as source of carbon displayed maximum lipid production (58.31%). Earlier researchers found comparable or lower lipid production utilizing different wastes by *R. glitinis* such as starchy waste water 35% (Xue et al., 2010), food waste leachates 47–49% (Johnravindar et al., 2018), rice residue 24.26% (Srisuwan et al., 2016). Gen et al. (2014) utilized processed cassava starch while in the present study, the waste part of cassava was used which makes the process cost effective and also take care of food vs fuel debate. Moreover, the strain *Rhodosporidium toruloides* used by Gen et al. (2014) did not produce amylase, therefore, amylase was also added during fermentation produced by another yeast *Saccharomycopsis fibuligera* A11-c. In our study *R. glutinis* itself produced high amount of amylase, so there was no need of supplementing additional enzyme for saccharification of starch into monomeric form. In present study, the overall C/N ratio(28:1) was also supportive for the yeast growth as well as for oil production in minimal media containing real life Cassava waste. Patel et al. (2017) also produced biodiesel from pulp and paper industry waste material which has minimal nutrients.

In earlier studies, lipid production was found to be affected by the pH of the medium and in this study, the most suitable pH was found to be 5.45. Similarly, pH 5–5.5 was reported to be optimum for *Aspergillus awamori* (Venkata and Venkata, 2014), *Penicillium brevicompactum* and *Trichoderma viride* (Ali et al., 2017; Ali and El-Ghonemy, 2014) for lipid production.

The incubation time is also a very important factor for lipid production. In present research, optimized time was 3.18 days or 75.6 h which is supported by El-Fadaly et al. (2009) who also reported 3 days (72 h) to yield 2.2 g/L oil from *Cryptococcus curvatus*. Ali et al. (2017) and Ali and El-Ghonemy (2014) reported 5–6 days for maximum oil production.

In optimization studies, the actual values supported the predicted values and effectiveness of the experimental model design was validated. A 9% increase in lipid production was achieved when compared to its basal medium. The experimental design RSM satisfy the

optimization of many microbial processes and minimize the fermentation period (Rocky-Salimi et al., 2011; Chang et al., 2006).

In our earlier study, several oleaginous yeast were found to produce lipid on various commercial starches as carbon source after 8 days incubation (Chaturvedi et al., 2018a) but in this study, maximum lipid was obtained within 3 days due to optimization which reduces the energy consumption needed for maintenance of fermentation conditions. The result also revealed the significance of RSM analysis which not only successfully increased the lipid content (0.073 g lipid/g of substrate to 58.31% dry biomass) but also further reduced the fermentation period by 5 days. The GC-MS based lipid profiling revealed the presence of ethyl-13 methyl tetradecanoate ($C_{17}H_{34}O_2$), oleic acid ($C_{18}H_{34}O_2$), hexadecanoic acid ($C_{18}H_{36}O_2$) and octadecanoic acid ($C_{20}H_{40}O_2$) as major fatty acids as reported by the earlier researchers (Tanimura et al., 2014; Saenge et al., 2011).

This study suggests an alternative route for utilization of cassava peel, an agro-industrial processing waste, reducing environmental problem as well as lipid production cost. Production of renewable environmentally safe bio-fuel as well as agro-industrial waste management leads in the direction of green energy and sustainable development. Scale up of the lipid production by *R. glutinis* in a bioreactor also leads to around 9% increase in lipid content as compared with batch studies. There have been many diverting competitive pathways such as melvanoic pathway towards carotene production which reduces the production of fatty acid due to flux diversion. Still this process is unique because cassava starch has been used without pretreatment higher oil was produced within short duration of 3 days making this research viable alternative for biodiesel production.

5. Conclusions

The production of biodiesel from starchy waste not only suggests a meaningful utilization of waste biomass but also helps in developing an economical process. The present study suggest use of starchy waste material as a source of carbon for the production of environmental friendly as well as cost effective biodiesel production process without pre-treatment of starchy substrate.

Statistical optimization and scale up in a bioreactor resulted in the accumulation of 58.31% of lipid by the *R. glutinis* within 3 days. Five lesser days translate in considerable energy saving in commercial level setting making the overall process highly cost effective.

Declarations

Ethics approval and consent to participate

This study did not involve any human participants nor animal studies.

Consent for publication

The authors declare that the research work was conducted, without any commercial or financial relationships, which may be construed as potential conflicts of interest. We confirm that no part of this work has been submitted in any other journal and the authors have no conflict of interest. All the authors have consented to the submission of this manuscript. All data generated are analyzed during this study are included in this published article (and its supplementary information files).

Availability of data and materials

The datasets supporting the conclusions of this article are included in the main manuscript. The authors promise to provide any missing data on request.

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Authors contributions

Shivani Chaturvedi along with Rameshwar Tiwari conducted the experiments and wrote the manuscript. Lata Nain and Sunil K. Khare provided advice regarding the experimental design and data analyses. All the authors read and approved the final manuscript.

Conflicts of interests

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

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