JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1383-1388

Activity Guided Optimization of Medium Components for Enhanced Production of Vinca Alkaloids by Endophyte

A. Garg, T. Saify, Gary Strobel, H. Kachchhava, R.T. Rowlands, N. Parikh, B.D. Patil and R. Srivastava

RPG Life Sciences India. 2702/A GIDC Ankleshwar Gujarat, India.

(Received: 18 April 2015; accepted: 06 May 2015)

An endophyte isolated and identified to produce Vinca alkaloid is evaluated with various media combinations for enhanced production. Commercially used carbon and nitrogen sources in various combinations were tried along with combinations of macro elements, hormones and inducers. The best media recipe indicated an enhanced titer of 115µg/L **against the control titer of** 80µg/L.

Key words: Endophyte, Vinblastine, Vincristine, Vinca, Catharanthus, Media Optimization.

Vinca alkaloids are one of the most effective anticancer molecules derived from the plant *Catharanthus roseus*. The yield of the molecule from the plant is very low owing to which their remains a scarcity of the molecule leading to high cost and unavailability. (Singh and Satyanarayana, 2006) A process employing endophyte to produce such molecules in microbial fermentation could bridge the demand-supply gap of the molecule.

Since, screening of this endophyte was done on basic Potato Dextrose medium where the endophyte represented 80ug/L of activity, (A. Garg et.al 2014) their remains an ample scope to develop the best suited recipe for the endophyte to yield better titers.

For an industrial fermentation process fermentation medium and fermentation process condition plays an critical role because they effect the formation, concentration and yield of a particular fermentation end product thus effecting the overall process economics therefore it is important to consider the optimization of fermentation medium and process conditions in order to maximize the profits from fermentation process (Schmidt, 2005). The aim of our study was to develop a new medium for cost- effective production of Vinca alkaloids with this endophyte.

* To whom all correspondence should be addressed.
E-mail: ankur.garg@rpgls.com

MATERIALS AND METHODS

Control medium and conditions

The endophytes were maintained on PDA slants. The production of Vinca alkaloids was done in three steps of liquid medium in which the vegetative medium was incubated in shake flask for 48hours in rotary shaker at 28^0C at 240 RPM. 10% of the vegetative medium was transferred to fresh vegetative medium after 48 hours and was placed in rotary shaker for another 24 Hours.

After 24 hours the production medium (Potato Dextrose broth) was inoculated with 10% of the vegetative medium and was incubated for 240 Hours. After 168 hours, the cells were harvested by centrifugation and were extracted in methanol for HPLC estimation.

Medium component and concentration optimization

The control starting medium potato dextrose broth was altered with various carbon

and nitrogen sources as mentioned in Table -01 and 03. The design of experiment was done keeping one change at one time criteria. The best combination was then evaluated with varying concentration of component to evaluate the most suited concentration for maximum production, as mentioned in Table -02 and 04.

Similar to Carbon and nitrogen microelements like phosphate, ammonium, magnesium and iron were also evaluated and optimized for increased Vinca production. Amino acids, inducers and growth hormone also indicated merits after evaluation.

Media Optimization

It is known that each strain behaves differently and have different requirements for major and minor constituents. In order to achieve higher titers of vincristine and to have most optimized up-scalable, commercially viable medium the potato dextrose broth was modified using "One change at a time" approach. In all the medium optimization experiments Potato dextrose broth (PDB) was kept as positive control to assess the percent improvement of individual change over the parent medium.

Carbon

Source optimization

In a series of three experiments, 15 carbon sources were examined, including polysaccharides, oligosaccharides, monosaccharides, and lipid materials. All carbon sources were tested at concentration of 20 Gms/L in Potato Dextrose broth medium, as replacements for Dextrose. Five carbon sources supported growth and Vincristine production (Table). The best carbon sources for vinblastine production by *endophyte* R1/E-45/N-102 were Malto Dextrin, dextrin white and soluble starch, representing **22.5, 12.5 and 2.5;** percent improvement respectively **(Table -01)**.

Lipid materials failed completely to support growth and Vinca alkaloid production. Although with Maltose and Glucose significant rise in Packed Cell Volume (PCV) was observed but these carbons were not found to support the secondary pathway of metabolite production.

Optimization of Malto dextrin concentration

One of the best carbon sources for *Vincristine* production on a volumetric as well as a specific basis was found to be Malto Dextrin (Table 02*)*. It was tested at different concentrations with the higher producing *endophyte* R1/E-45/N-102. As mentioned in the table below the most optimum concentration of malto dextrin was found to be 40 grams per liter for the production of Vincristine. Further higher concentrations do not reflect any benefit on the titers.

Table 1. Optimization of carbon source for enhanced production of Vinca alkaloids

S. No	Carbon Source	Harvest at 168 Hrs			% improve -ment
		pH	PMV	Titer (µg/L)	
1	PDB Control	5.8	24	80	*
2	D-Fructose	4.2	14	28	*
3	Dextrose	5.1	20	56	*
4	Malto Dextrin	6.2	34	98	22.5
5	o(+) Galactose	5.2	22	56	*
6	D-Mannitol	5.6	22	52	*
7	Dextrin white	6.1	32	90	12.5
8	D(+)Xylose	8.2	16	20	*
9	Soluble starch	5.4	28	82	2.5
10	Soya oil	4.6	12	12	*
11	Coconut oil	4.8	12	12	*
12	Glucose	8.1	46	52	*
13	Glycerol	5.8	20	24	*
14	Sesame oil	8.4	12	20	*

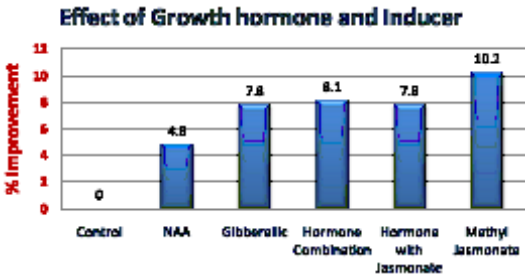

Table 2. Optimization of Malto dextrin concentration for enhanced production

S. No	Malto Dextrin Concentration (Gms/L)	Harvest at 168 Hrs			% improve -ment
		pH	PMV	Titer (µg/L)	
1	10	5.2	16	32	-66.0
2	20	6.2	34	94	Control
3	30	6.2	40	114	21.3
4	40	5.8	36	110	17.0
5	50	4.6	32	84	-10.6
6	60	4.5	28	65	-30.9

Nitrogen

Source optimization

To potato dextrose broth medium containing 30g/l of Malto dextrin in place of dextrose, Thirteen, different nitrogen equivalent sources were added, one-at-a-time, as replacements potato infusion. Also, medium without potato infusion was studied. As illustrated in **(Table-03)**, the medium having no nitrogen source was found unstable for vincristine production.

Moreover, nearly with all tested nitrogen sources, Vincristine titer has clearly dropped except for the source soya flour, where a considerable rise of **13.68%** in titers of Vinblastine was seen with respect to potato infusion.

Table 3. Optimization of Nitrogen source for enhanced production of Vinca alkaloids

S.No	Nitrogen Source (10 Gms/L)	Harvest at 168 Hrs			% improvement
		pH	PMV	Titer (µg/L)	
1	Potato infusion with 30 gm/L malto dextrin	5.8	24	95	*
2	None* (Without Nitrogen)	6.3	08	12	*
3	Ammonium sulphate	6.4	12	15	*
4	Ammonium Chloride	4.9	10	12	*
5	DiammoniumHydrogen Phosphate	6.0	16	18	*
6	Sodium Nitrate (Sigma)	7.2	16	12	*
7	Urea (HiMedia)	7.1	28	22	*
8	Yeast Extract (HiMedia)	6.9	32	36	*
9	Beef Extract (HiMedia)	5.8	30	70	*
10	Soya Peptone (HiMedia)	7.3	44	75	*
11	Casein (HiMedia)	6.3	26	48	*
12	Soya flour (HiMedia)	6.2	44	108	13.68%
13	Liver extract (HiMedia)	6.5	38	68	*

Table 4. Optimization of soya flour concentration for enhanced production of Vincristine

S. No	Soya Flour Concentration (Gms/L)	Harvest at 168 Hrs			% improve -ment
		pH	PMV	Titer (µg/L)	
1	5	6.2	36	91	*
2	10	6.2	44	108	Control
3	15	6.8	44	96	*
4	20	5.2	44	84	*
5	25	6.4	46	65	*
6	30	8.2	52	46	*

Optimization of Soya flour concentration

Since in the above set of experiments only 10 grams/liter of soya flour was tried, it was necessary to optimize the optimum concentration of soya flour for highest production of vincristine. For this, 06 different concentrations from 05 Grams/ liter to 30 grams/l were tried increasing 05 grams/l in each set. It is found that the concentration 10

Table 5. Optimization of phosphate concentration

S. No	Concentration (mM)	Harvest at 168 Hrs			% improve -ment
		pH	PMV	Titer (µg/L)	
1	00	6.4	44	104	Control
2	10	6.5	34	85	*
3	25	7.1	40	68	*
4	50	7.6	52	45	*
5	100	8.2	54	28	*
6	200	8.8	54	20	*

Table 6. Optimization of Ammonium concentration

S. No	Concentration (mM)	Harvest at 168 Hrs			% improve -ment
		pH	PMV	Titer (µg/L)	
1	00	6.4	44	104	Control
2	10	6.4	44	95	*
3	25	6.5	50	26	*
4	50	6.6	50	24	*
5	100	7.2	32	06	*
6	200	8.4	18	02	*

grams/l is the most optimum concentration giving the best titer of 108 µg/L of Vinblastine**.(Table – 04)** It is also observed that with increasing concentration, although the PMV has increased but the titer of Vinblastine is not increasing. This may be due to the repression effect of nitrogen on the secondary metabolite pathway of vincristine production.

Trace elements

Source optimization

In this study the effects of phosphate, ammonium, magnesium and iron on formation of Vincristine by the best mutant is assessed as detailed in the table below.

Phosphate nutrition

In optimized medium (30 GPL malto dextrin and 10 GPL soya flour), phosphate was added as K_2HPO_4 (2 GPL), and KH_2PO_4 (2 GPL), a total phosphorus concentration of 26.5 mM. To determine whether phosphate exerts control of Vinblastine production, the concentration of K_2HPO_4 was used from 0 to 200 mM.

Table-05 as above represents that the set without phosphate represented better titers as compared to the sets with phosphate salts. It is also observed that with increasing concentration the packed cell volume has increased significantly from 44 to 54 suggesting that the phosphate, although have positive influence on the growth of the strain but have negative effects on the secondary metabolite pathway of vincristine.

Ammonium nutrition

A negative effect on Vinblastine formation was found upon addition of all concentrations of NH_4Cl from 5 mM to 200 mM **(Table-06)**. Growth, on the other hand like phosphate addition, was stimulated by NH_4Cl up to 25 mM.

Table 8. Optimization of Ferrous ion concentration

S. No	Concentration (mM)	Harvest at 168 Hrs pH	PMV	Titer (µg/L)	% improve -ment
1	00	6.4	44	104	Control
2	0.01	6.45	38	102	*
3	0.05	6.86	40	92	*
4	0.10	6.92	36	88	*
5	0.50	7.25	34	34	*

Magnesium Nutrition

To study possible Mg control of Vincristine formation, increasing concentrations of $MgSO_4.7H_20$ were tried. It is clear that Mg controls Vincristine production. Production was optimal at 0.01 mM $MgSO_4$.7H20 (2.5 mg/L). Increase of 7.7% was observed. Further increases with $MgSO_4$ up to 1 mM increased growth but interfered with vincristine production **(Table-07)**.

Iron Nutrition

To estimate the possibility of Iron (Fe) control on Vinblastine formation, increasing concentrations of $FeSO_4$ were tried. It is clear that Fe+ ions do not control Vincristine production. Varying $FeSO_4$ concentration does not represented any merits. **(Table-08)**

Optimization of Amino acids

Source optimization

Vinca alkaloids as apparent of the pathway are the products derived from amino acid L-Methionine as precursor. Various amino acids were tested to the most optimized medium and it was found that L-Methionine represented merits over control medium as compared to L-Proline and L-Lucien used for the study.

Table 7. Optimization of Magnesium concentration

S. No	Concentration (mM)	Harvest at 168 Hrs pH	PMV	Titer (µg/L)	% improve -ment
1	00	6.4	44	104	Control
2	0.01	6.3	42	112	7.7%
3	0.05	6.3	44	100	*
4	0.10	6.6	42	102	*
5	0.50	6.6	44	90	*
6	1.00	7.2	46	81	*

Table 9. Optimisation of L-Methionine concentration

S. No	Concentration (Grams/Liter	Harvest at 168 Hrs pH	PMV	Titer (µg/L)	% improve -ment
1	00	6.32	42	105	0.0
2	0.5	6.40	40	101	-3.8
3	1.0	5.92	36	106	1.0
4	2.5	5.85	36	114	8.6
5	5.0	5.64	34	104	-1.0
6	7.5	5.23	32	84	-20.0
7	10.0	5.02	28	62	-41.0

Table 10. Optimization of Inducer

S.No	Concentration (Grams/Liter)	Harvest at 168 Hrs			% improvement
		pH	PMV	Titer (µg/L)	
1	Control	6.38	40	104	Control
2	Methyl Jasmonate (0.05%)	5.24	32	58	-44.23
3	Methyl Jasmonate (0.01%)	6.01	36	86	-17.31
4	Methyl Jasmonate (0.005%)	6.50	42	114.8	10.4
5	Methyl Jasmonate (0.001%)	6.4	40	102.5	

Table 11. Optimization of Growth Hormone

S. No	Concentration (Grams/Liter)	Harvest at 168 Hrs			% improvement
		pH	PMV	Titer (µg/L)	
1	Control	6.4	40	105	0
2	NAA (0.1%)	6.35	42	110	4.8
3	Gibberellic Acid (0.05%)	6.42	40	113	7.8
4	NAA (0.08%) + Gibberellic Acid (0.02%)	6.5	40	114	8.1
5	NAA (0.08%) + Gibberellic Acid (0.02%) + Methyl Jasmonate (0.005%)	6.5	40	113	7.8
6	Methyl Jasmonate (0.005%)	6.42	42	116	10.2

Optimization of L-Methionine concentration:

To optimize the optimum concentration of L-Methionine for Vincristine production in the optimized medium various concentration from 0.5 Grams/Liter to 10 Grams/ Liter were tested. It was found that L-Methionine at a concentration of 2.5 GPL in the optimized medium represented 8.6% increase over the optimized control medium. **(Table-09)**

Optimization of inducers.

Various inducers that are reported in literature were tried and found that methyl Jasmonate represented enhancement in titers. With further concentration optimization it is established that with 0.005% of Methyl Jasmonate the enhancement was ~10% as compared to control of without Methyl Jasmonate.

As represented in **Table 10** it is also observed that concentration lower than 0.005% represented no effect on the titers whereas the concentration 0.01% represented feedback inhibition and the titer decreased to 86 µg/L as compared to control of 104 µg/L.

Optimization of Growth Hormone

Both Auxin and Gibberellins were evaluated of various concentrations in optimized medium. NAA 0.1% and Gibberellic acid 0.05% represented best results of 5% and 8% respectively. Both these active growth hormones were then enumerated for their synergistic effect and was found that 0.08% of 1-Naphthaleneacetic acid (NAA) combined with 0.02% of Gibberellic acid represented enhancement of 08% against control without hormones.

Growth hormone combined with Methyl Jasmonate did not represent further added enhancement in titers. As represented in table it is also observed that methyl Jasmonate alone, works better with the optimized medium as compared to growth hormone representing 2% increase over the combination as well as the individual performances of Growth hormones. **(Table-11)**

CONCLUSION

After optimization of complete media recipe it is evident that a significant increase in overall productivity of Vinca alkaloid is achieved. Control titers which were in the range of 80µg/L (±05) have increased to 115 µg/L (±05). This rise of 44% in production to the control titers is noteworthy and indicates that with further optimization of growth conditions and inoculums the process could be a viable successful and feasible process for future production of Vinca alkaloids through the fermentation route.

REFERENCES

1. Schmidt, F.R., Optimization and scale up of industrial fermentation processes. *Applied Microbiol. Biotechnol.*, 2005; **68**: 425-435.
2. Singh, B. and T. Satyanarayana, A marked enhancement in phytase production by a thermophilic mould Sporotrichum thermophile using statistical designs in a cost-effective cane molasses medium. *J. Applied Microbiol.*, 2006; **101**: 344-352.
3. Garg, T. Saify, N.M Khan, V. Kucheria, C. Prajapati, H. Kachchhava, D. Pote, and R. Srivastava. Endophyte - Plant to Plants. *Journal of Pure and Applied Microbiology* 2014; **8**(1).