ISSN: 0975-7538
Research Article

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Process optimization for the cultivation of anti-cancer *Pseudomonas aeruginosa* (MTCC 647) bacterial strains

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ABSTRACT

Cancer is one of the most dangerous diseases worldwide. Bacterial products such as proteins and endotoxins (Lipo polysaccharides) have been tested for cancer treatment. In this work an effort was made to increase the biomass and total protein content of *Pseudomonas aeruginosa* extract by using Asparagine proline broth and optimum growth conditions. Bacterial strains of *Pseudomonas aeruginosa* MTCC no 647collected from MTCC, Chandigarh. AP broth was optimized by addition of copper sulphate, methylamine and aluminum sulphate. Modified Asparagine proline broth (AP1 to AP5) was prepared and the effect of graded amounts of copper sulphate and aluminum sulphate were analyzed. Asparagine proline broth supplemented with 0.03% of copper sulphate and 0.02 % of aluminum sulphate (AP3) was selected as it has shown high cell growth rate. Influences of various culture conditions such as temperature, P^H, Incubation time on cultivation of *Pseudomonas aeruginosa* in modified Asparagine proline broth were studied. The highest protein production was achieved at 43ºC, at pH 8 for a period of 48hrs.

Keywords: Asparagine proline broth; Bacterial proteins; biomass; Media optimization and *Pseudomonas aeruginosa.*

INTRODUCTION

Cancer is one of the most dangerous diseases worldwide. Cancer development is a multi-factorial process (Shappell et al., 2004; Lakritz et al., 2014), its evolution depending on the micro and macro environment**.** According to International Agency for Research on Cancer (IARC) (WHO) Global burden rises to 14.1 million new cases and 13.3 million people are likely to die annually of cancer by 2030.

Conventional anticancer therapies are affected by development of drug resistance and side effects in patients with advanced solid and liquid borne tumors. Hence, there is a strong need of alternative cancer therapies. Nowadays, multi-targeted approaches have gained greater importance in cancer therapy. New generation of drugs is urgently needed to achieve the concept of multi-targeted therapy (Chakrabarty AM et.al, 2014).

Bacterial products such as proteins and endotoxins (Lipo polysaccharides) have been tested for cancer treatment. Bacterial proteins are used for tumor destruction. Purified bacterial products are also gaining relevance as new classes of bioactive products to treat and prevent cancer growth and metastasis. Some of these products have proven to cause significant and promising results, such as tumor regression through growth inhibition, cell cycle arrest or even apoptosis induction (Fialho AM et.al, 2012). In future, cancer vaccines can be produced based on proteins and immunotoxins of bacterial origin (Bernardes N et al., 2010).

Bacteria can be used as vectors to deliver anticancer drugs specifically to tumor cells. Spores of anaerobic bacteria can be used for the aforementioned strategies because only spores that reach an oxygen starved area of a tumor will germinate, multiply and become active. The use of genetically modified bacteria for selective destruction of tumors, and bacterial gene-directed enzyme prodrug therapy have shown promising potential.

Bacterial redox protein such as Azurin complex with p53 protein, stabilize and increase its intracellular level and produce apoptosis. Azurin protein transduction domain specifically penetrates cancer cells and produce cytostatic and cytotoxic effects (Divya K. Damania, 2016).

Proteins of *P. aeruginosa* strain reduce toxic effects in regression of cancer treatment. Previous study reveals that the blue copper protein azurin with cytochrome c can be synthesized from different microbial sources, specifically from *P. aeruginosa* (Parr SR et.al.1979; Go-

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Received on: 10-04-2017
Revised on: 23-05-2017
Accepted on: 28-05-2017

to M et.al, 2003). Recently, azurin was shown to be capable of cause a decrease in P-cadherin levels, without interfering with E-cadherin levels, in three different breast cancer cell lines, including MCF-7/AZ.Pcad (Bernardes N et.al, 2013). Azurin involved in the dentrification of *Pseudomonas aeruginosa*. Rusticyanin consist of single polypeptide chain of 159 amino acids residue with Cu at the active site (De Rienzo F et al., 2000).

Hence, in this work an effort was made to increase the biomass and total protein content of *Pseudomonas aeruginosa* extract by using Asparagine Proline Broth and growth conditions (Gupta R et al., 2003).

Asparagine proline broth is recommended for cultivation of *Pseudomonas aeruginosa*. The medium is recommended by BIS. Asparagine Proline Broth contains both the enantiomeric forms of Asparagine, which is readily utilized by *Pseudomonas* for their growth. Phosphates and sulphates provide the ions for the growth as well as buffer the medium to promote the growth of the organism.

MATERIALS

Bacterial strains of *Pseudomonas aeruginosa* MTCC no 647collected from MTCC, Chandigarh. Media such as Luria-Bertani (LB) broth and Asparagine proline (AP) broth were selected for the study.

METHOD

Asparagine-proline (AP) broth was used for the study to increase the yield of biomass and azurin protein content from selected bacterial culture. Initially, 50 ml of LB medium is taken in 250 ml Erlenmeyer flasks and inoculated with culture and shaken overnight at 250 rpm, 37 °C to prepare seed culture. From shake flask culture, 2 ml of the seed culture was transferred into 250 ml of the respective medium in a 1 L Erlenmeyer flask and incubated at 37 °C, 300 rpm, for 12 h. After 4 h of incubation, 0.5 mM IPTG was added to the media to induce growth. Further all the cultures were incubated for a period of 8 h. Samples were collected for measurement of dry cell weight and total protein yield. The Lowry method is used for total protein estimation (Parr SR., 1976). The color density was recorded using spectrophotometer at 660 nm and compared to standard curve of protein (BSA 1mg /ml).

Optimization of culture media and culture conditions

Modification of Asparagine-proline (AP) broth

Asparagine proline broth was modified by the addition of graded amount of substances such as copper sulphate, methylamine and aluminum sulphate to increase the yield of total protein fraction.

Effect of copper sulphate

Graded amount copper from 0.01% to 0.05% was added to the media to induce the yield of azurin protein.

Effect of methylamine

0.1%methylamine was added to Asparagine proline

broth. It serves as a source of carbon, energy and/or nitrogen for a wide range of bacteria.

Effect of aluminum

The genes encoding blue copper-binding proteins are induced by Aluminum mediated oxidative stress. Hence graded amount of Aluminum salt was added to trigger the azurin protein production.

Optimization of culture conditions

Varying culture parameters were studied to increase the yield of total protein fraction in Asparagine proline broth such as effect of temperature, pH, and incubation time (Davis KE et al., 2005). Influences of these parameters were used to determine the total biomass and total protein fraction at 660 nm.

The effect of temperature on protein production was determined at varying temperatures *viz.* 30, 35, 40 and 45ºC maintained during the incubation period of inoculums used for protein production. To 100 ml production medium (pH: 7), 1% inoculum was added and incubated for 24-48h. Effect of pH on protein production by the bacterial cultures was determined at optimized temperature for 24-48 h while varying the pH values of the medium *viz.* 2, 4, 6, 8 and 10. Effect of incubation time was evaluated at optimized temperature and pH. Production flasks were subjected to different incubation time *viz.* 0, 12, 24, 48, 72 and 96 hrs.

Cell dry weight measurement

From inoculated flasks, the contents each containing 50 ml were transferred to a cooling centrifuge **(**Cooling Micro centrifuge Model C-24BL, Remi) in pre- weighted centrifugation tubes of 50 ml volume at 5000 rpm for 15 minutes. The cell precipitates were collected, washed with sterile 0.9% saline solution and re-centrifuged. The supernatants were discarded and the centrifugation tubes were dried in an oven (Hot air oven Biotechnics, India) at 100°C till constant weights were obtained. The weight of the dried cells was measured by calculation of the difference between the weight of centrifugation tubes before and after dryness.

Ultra Sonication and total protein estimation

Two ml of bacterial culture was taken in five eppendorf tubes and centrifuge at 10000 rpm for 5 minutes and the cell pellets is were kept in ultrasonic wave generator After sonication, the eppendorf tubes were centrifuged at 10000 rpm for 5 minutes. Supernatant is taken and the amount of protein in the sample is determined by Lowry's method.

Fed-batch cultivation *Pseudomonas aeruginosa* strains

After the shake flask culture, the overnight grown culture in Optimized AP medium was further grown in a 3

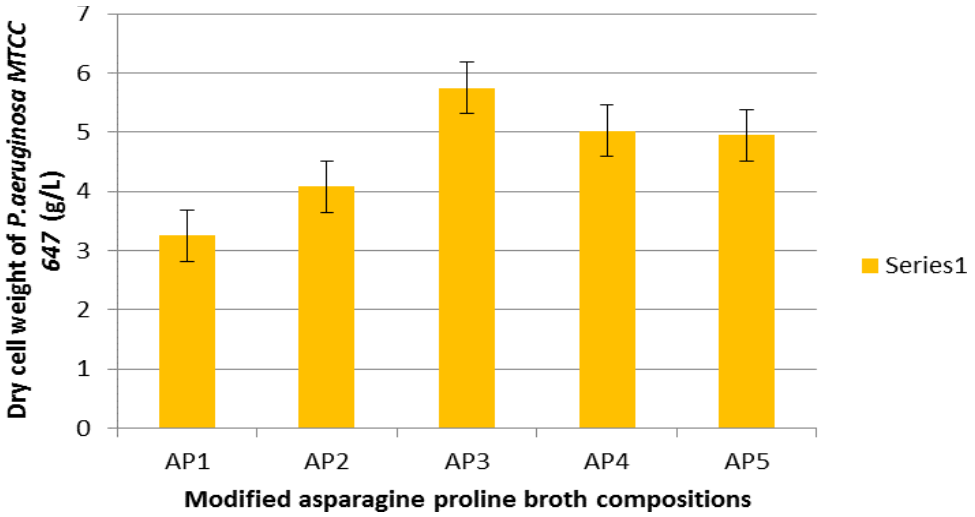

Figure 1: Effect of modified Asparagine proline broth media on dry cell weight of Pseudomonas aeruginosa (MTCC 647)

Table 1: Composition of Asparagine proline broth

S.No	Ingredients	gms / Litre
1	DL-Asparagine	2.000
2	L-Proline	1.000
3	Di potassium phosphate	
4	Anhydrous	1.000
5	Magnesium sulphate	0.500
6	Potassium sulphate	10.000

**Formula adjusted, standardized to suit performance parameters

Table 2: Dry cell weight (g/L) of Pseudomonas aeruginosa (MTCC 647) in modified Asparagine proline broth media

Modified Asparagine proline broth	Composition of Asparagine proline broth	Dry cell weight(g/L) *Pseudomonas aeruginosa* (MTCC 647)
AP1	APbroth+0.01%CuSo4+0.1% methylamine+0.01% aluminium sulphate	3.25
AP2	APbroth+0.02%CuSo4+0.1% methylamine+0.02% aluminium sulphate	4.08
AP3	APbroth+0.03%CuSo4+0.1% methylamine+0.03% aluminium sulphate	5.75
AP4	APbroth+0.04%CuSo4+0.1% methylamine+0.01% aluminium sulphate	5.02
AP5	APbroth+0.05%CuSo4+0.1% methylamine+0.01% aluminium sulphate	4.95

L bioreactor by fed-batch cultivation to achieve higher cell densities. After approximately 18 hrs of bioreactor

cultivation (~4 after induction), final biomass yield was measured. The bioreactor provides for control of culture pH, effective aeration, feeding with a carbon source, antifoam, and a nitrogen source, and also maintains culture sterility.

Statistical analysis

Data analysis was done using SPSS software version 10 (windows). The results were represented as mean ± SE for three replicates.

RESULTS

Pseudomonas aeruginosa culture was grown in Asparagine proline broth. In shake flask culture, final cell concentration was found to depend upon the media used. AP broth was optimized by addition of copper sulphate, methylamine and aluminum sulphate. Modified Asparagine broth (AP1 to AP5) was prepared and the effect of graded amounts of copper sulphate and aluminum sulphate were analyzed.

In each composition of AP media, cell biomass was measured and reported in table 2. Asparagine proline broth supplemented with 0.03% of copper sulphate and 0.02 % of aluminum sulphate (AP3) was selected as it has shown high cell growth rate (Figure.1). Influences

Table 3: Effect of PH on total protein fraction of Pseudomonas aeruginosa (MTCC no 647)

S.NO	pH	Total protein content(mg/ml)
1	2	0.53±0.01
2	4	1.42±0.04
3	6	1.83±0.03
4	8	2.62±0.03
5	10	2.11±0.04

Mean ± standard error (SE) for three replicates

Table 4: Effect of temperature on total protein fraction of Pseudomonas aeruginosa (MTCC no 647)

S.NO	Temperature in ^{0}C	Total protein content(mg/ml)
1	30	2.03±0.05
2	35	2.25±0.06
3	37	2.68±0.05
4	40	2.70±0.04
5	43	2.90±0.05
6	45	1.26±0.04
6	50	1.04±0.04

Mean ± standard error (SE) for three replicates

Table 5: Effect of Incubation time on total protein fraction of Pseudomonas aeruginosa (MTCC no 647)

S.NO	Incubation time (hrs)	Total protein content(mg/ml)
1	12	2.28±0.05
2	24	3.64±0.06
3	48	3.94±0.05
4	72	4.03±0.06
5	96	3.23±0.06

Mean ± standard error (SE) for three replicates

of various culture conditions such as temperature, p^H, Incubation time on cultivation of *Pseudomonas aeruginosa* in optimized Asparagine proline broth were studied to determine the total biomass and total protein fraction at 660 nm (Khusro A et al., 2014).

The findings were reported table 3, 4, and 5. The highest protein production was achieved at 43ºC. Highest biomass and protein content at pH 8 while lowest biomass was produced at pH 2. A gradual increase in biomass content observed from 12 h towards to 72 h. After 72 h of incubation time, a decrease in the biomass was observed. Overall the highest biomass was observed when the organism grown in Asparagine proline broth at 43^{0}C, at a slightly alkaline pH of 8 for a period of 72 hours. Increase in yield of the biomass was observed when culture grown in fed batch bioreactor.

DISCUSSION

Bacteria and its proteins show selectivity for tumor tissues. Bacteria and their spores also serve as ideal vectors for delivering therapeutic proteins to tumors. Peptides of *Pseudomonas aeruginosa* leads to Apoptosis in Human Cancer Cell Lines. In this work, it was proposed to increase the biomass and protein content of *Pseudomonas aeruginosa* (Goto M et al., 2003).

Pseudomonas aeruginosa (MTCC NO 647) was cultivated in asparagine-proline (AP). Asparagine proline broth allows high growth rate as it contains a strictly mineral base with asparagine as a sole source of nitrogen and glycerol as the carbon source. The potassium salts act as a buffer system and magnesium sulfate is a source of magnesium ion required in a large variety of enzymatic reactions, including DNA replication and acts as a buffer. AP broth was optimized by addition of copper sulphate, methylamine and aluminum sulphate (Su X et al., 2015). Graded amounts of copper increased the yield of biomass and protein content of the cell extract. Oxidative stress induced by toxic levels of metal ions such as aluminium and other stress conditions triggers genes such as blue-copper proteins. Hence trace amount of aluminium salt was added to the Asparagine proline broth. Aluminiun induced oxidative stress induced the genes encoding blue copper proteins of Pseudomonas aeruginosa and increased the total protein fraction (Shah AH et al., 2008).

Shake flask culture was developed initially using seed culture of bacteria. Asparagine proline broth medium was chosen for the cultivation of *pseudomonas aeruginosa* strains. Modified asparagine-proline broth supplemented with copper sulfate, methyl amine and aluminium salt has increased the biomass and protein fraction. Highest biomass was produced in AP3 media which was supplemented with 0.03% $CuSo_4$, 0.1%methylamine and 0.03%aluminium sulphate. Around 5.7 gm/L of biomass was produced in AP3 media. Extracellular soluble protein in culture was esti-

mated by the Lowry's method using bovine serum albumin (BSA) used as a standard.

Effect of various culture parameters such as temperature, $P^{H,}$ incubation time was studied (Scervino JM et al., 2011). Temperature effects the growth of bacteria in soil, aquatic environment and in air. Bacterial biomass production correlates the best with temperature rather than other factors. The optimal temperature for bacterial growth is 5 ^{0}C warmer than that of fungi (Pietikainen et al., 2005). As there is an increase in temperature, there was an increase in yield of the total protein content till 43°C. Biomass growth was optimum till 72 h of incubation. A decrease or increase in the incubation temperature leads to the lower growth of the organism. The pH of the culture medium directly influences the growth of microorganisms and the chemical processes that they perform. The optimum pH for protein production was determined by growing *pseudomonas* culture at pH 2, 4, 6, 8, and 10. The earlier studies revealed that an optimum pH range for the growth of bacterial strains is between 6.0 and 7. In case of *Pseudomonas* high biomass and protein content was observed at slightly alkaline pH. As incubation time increased, there was also a corresponding increase in biomass and protein fraction. Incubation period effected the growth of the culture (Kumar R and Vats R, 2010). Protein production began 12 h after shake flask culture and increased gradually to a maximum level of 4.03mg/mL at 72h thereafter, protein production decreased (Chikere CB et al., 2014).

CONCLUSION

Pseudomonas aeruginosa can be used for production of novel anti-cancer proteins. In the present study, asparagine-proline broth supplemented with copper sulfate, methyl amine and aluminium salt is chosen to increase the biomass and protein fraction. The optimum growth parameters for cultivating the organism and maximum protein content were determined. This study provides promising information on media optimization for economic production of *Pseudomonas aeruginosa* based proteins for exploring their anti - cancer activity.

CONFLICTS OF INTERESTS

All authors have none to declare.

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