Original Research Article

EFFECT OF DIFFERENT CARBON SOURCES AND GROWTH SUPPLEMENTS ON GROWTH AND BIOMASS PRODUCTION OF BIOINOCULANT *Azospirillum lipoferum* Az204

ABSTRACT

In this study the growth and cell yield of *Azospirillum lipoferum* Az204 was investigated in different carbon sources. Also the effect of adding growth promoting nutrients (0.1% of biotin or 0.1% yeast extract) along with the carbon sources was tested in Nfb medium. The study revealed the ability of *A. lipoferum* Az204 to grow in malic acid, glucose, succinic acid and glycerol. Although addition of 0.1% of biotin enhanced the cell number and biomass the growth in all carbon sources was at it's maximum in the presence of 1g L^{-1} of yeast extract. In glucose and yeast extract combination the strain reached maximum viable cell count of 11.8 log cfu ml^{-1} and biomass yield of 5.25 g L^{-1} at the end of 24 hours. It is followed by malic acid + yeast extract which showed cell count of 9.25 log cfu ml^{-1} and biomass yield of 4.13 g L^{-1} at 24 h time. Among all the carbon sources glycerol showed minimum growth. Glycerol combined with yeast extract produced viable cell count and biomass yield of 5.5 log cfu ml^{-1} and 1.82 g L^{-1} respectively. Succinic acid showed intermediate growth and when used along with yeast extract gave viable cell count and biomass yield of 8.22 log cfu ml^{-1} and 3.82 g L^{-1} respectively.

Keywords: (Azospirillum, cell growth, carbon sources, growth promoters, biomass)

1. INTRODUCTION

Bacteria of the genus *Azospirillum* are free living microbes that promote plant growth. *Azospirillum* is one of the most studied plant growth promoting bacteria.They have an impact on the growth and production of wide variety of plant species, which are important for both agricultural and environmental reasons [1]. *Azospirillum* has the ability to transform atmospheric nitrogen into ammonium under microaerobic conditions and low nitrogen levels, through the nitrogenase complex action. The role of *Azospirillum* in promoting plant growth including activities such as nitrogen fixation [2] and the synthesis of phytohormones, polyamines and trehalose is widely accepted. These bacteria secrete phytohormones such as auxins, cytokinins, and gibberellins that produce changes in plant root architecture, inducing the development of adventitious roots [3,4] and root hairs on their host plants which is beneficial due to root growth stimulation. The prevalence of the *Azospirillum* genus in agriculture is largely attributed to it's ability to fix atmospheric nitrogen in higher plants [5].

Much work has been done on *Azospirillum* inoculation effect on various crops that significantly improved yield and other growth parameters. But at the end inoculants made of viable cultures with high numbers are essential to bring out the desired effect in plants. Achieving high cell yields with higher growth rate is a crucial step in making these products commercially viable [6] as faster growth rate reduces expenses and production process can be economical. When it comes to biofertilizer production, the choice of carbon source plays a pivotal role in determining process yield and efficiency [7]. They also require growth promoting nutrients. Biotin, also recognized as vitamin B7, is a water-soluble essential vitamin crucial for the proper functioning of various organisms, spanning from bacteria to humans. Within eukaryotic cells, biotin plays a pivotal role as a coenzyme, particularly in the form of carboxylases. These enzymes facilitate critical biochemical reactions, including those involved in gluconeogenesis, amino acid breakdown, and the synthesis of fatty acids [8].Consequently, biotin plays a significant role in enhancing cellular metabolic processes related to carbohydrates, lipids, and proteins, thereby contributing to the acceleration of cell growth. Supplements like yeast extract can

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enhance the nutritional content of the medium by providing not only organic nitrogen and carbonaceous compounds but also growth-promoting substances [9]

The primary goal of this study is to select the most suitable carbon source and other nutrient supplements for incorporation into the existing growth media to enhance cell growth and biomass yield in the broth culture used for commercial inoculant production.

2. MATERIAL AND METHODS

2.1 Bacterial strain and culture conditions

Mother culture of *A. lipoferum* Az204 was obtained from the Commercial Bioferttilizer Production unit, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The strain was originally isolated from paddy crop and commercially mass produced for crop applications. A. lipoferum Az204 was cultured for 24 hours at 36± 1°C inn Nfb broth containing 1g L-1 of NH4Cl

2.2 Culture media

Nfb media was used for the study. The medium contains (g L−1): malic acid, 5.0; K2HPO4, 0.5; MgSO4.7H2O, 0.2; NaCl, 0.1; CaCl2. 2H2O, 0.02; micronutrient solution, 2 mL (CuSO4.5H2O, 0.04; ZnSO4.7H2O, 0.12; H3BO3, 1.40; Na2MoO4.2H2O, 1.0; MnSO4. H2O, 1.175. Complete volume to 1,000 mL with distilled water); bromothymol blue, 2 mL (5 g L−1 in 0.2 N KOH); FeEDTA , 4 mL (solution 16.4 g L−1); vitamin solution, 1 mL (biotin, 10 mg; pyridoxal-HCl, 20 mg in 100ml water); KOH, 4.5 g. [10]. The Nfb medium was added with 1g L-1 NH4Cl in all experiments. The growth of *A. lipoferum* Az204 in different carbon sources such as malic acid, succinic acid, glucose and glycerol was recorded. Also growth promoting nutrients such as 0.1% biotin or 0.1% yeast extract were used in combination with all the carbon sources. The treatments were, (i) Malic acid (ii) Glucose (iii) Succinic acid (iv) Glycerol (v) Malic acid + biotin (vi) Glucose + biotin (vii) Glycerol + biotin (viii) Malic acid + yeast extract (ix) Glucose +yeast extract (x) Succinic acid + yeast extract (xi) Glycerol + yeast extract. Vitamin solution was not added in treatments with carbon sources alone (I,ii,iii,iv)

2.3 Inoculation and growth conditions

A. lipoferum Az204 was grown in 100 ml medium for each treatment in 250 ml conical flasks. Flasks were inoculated with 5 ml fresh *A. lipoferum* Az204 cultures whose Optical Density at 540nm was 1 and were incubated at 36±1°C in shaking condition at 120 rpm .Samples were drawn at one hour interval till 12 h and then at 18 and 24 h of incubation, Optical Density at 540 nm and the culture pH were measured and viable counts and biomass estimation were performed. Viable populations were recorded as colony-forming units per millilitre (cfu ml-1) after incubating at 36ºC for 24 h on respective medium.

2.4. Biomass

For biomass estimation 50 ml of culture broth from each treatment was drawn after 24 h. biomass the culture broth is stirred inorder to suspend the culture evenly. The cells are separated from the culture broth by centrifuging them at 6000 rpm for 15 minutes. After discarding the supernatant the samples are placed in hot air oven and dried at 105º C for 24 h. The cell dry weight was expressed in g L^{-1} of the culture broth.

2.4 Statistical analysis

Each medium was tested in three replicates where a single erlenmeyer flask serves as a replicate. Data were analyzed by one-way ANOVA and then by Tukey's post hoc analysis set at $P< 0.05$.

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3. RESULTS

The carbon sources malic acid, succinic acid, glycerol, glucose in Nfb broth were screened for supporting the growth of *A. lipoferum* Az204 along with growth promoting nutrients like 0.1% of biotin or 0.1% of yeast extract. The growth results in terms of mean log cfu ml-1 were given in (Fig.1).

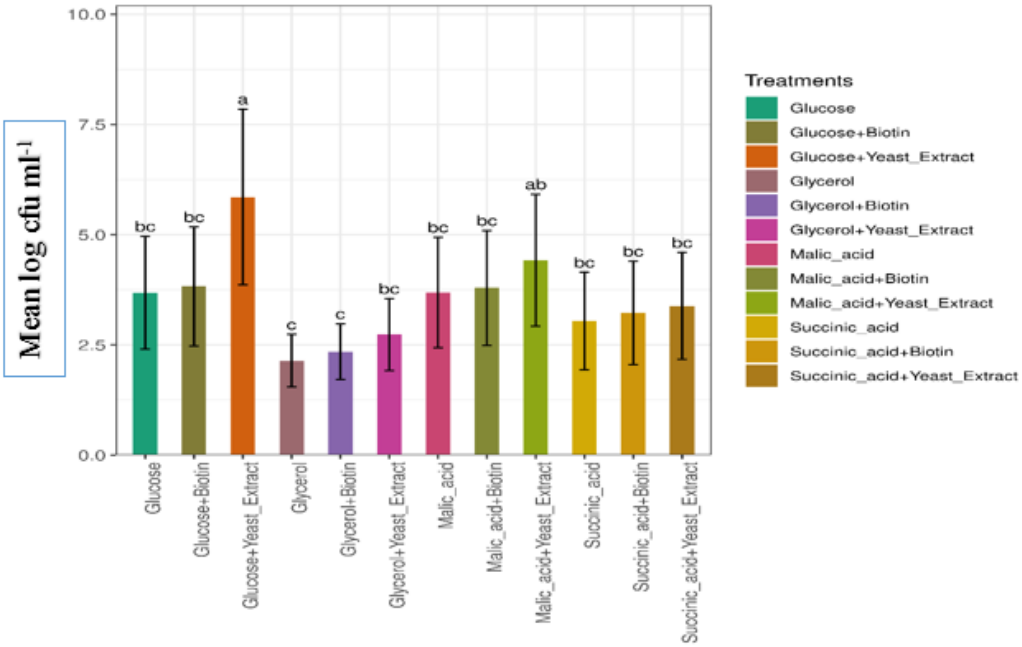

Fig 1. Growth of *A. lipoferum* Az 204 in media containing different Carbon sources and growth promoting nutrients.

(Since the P-value in ANOVA table is < 0.05, there is a significant difference between atleast a pair of treatments) For each carbon source, values denoted with different lower case letter differ significantly at P<0.05 by one-way ANOVA and Tukey's post hoc analyses.The bar represents the significant differences between the treatments.

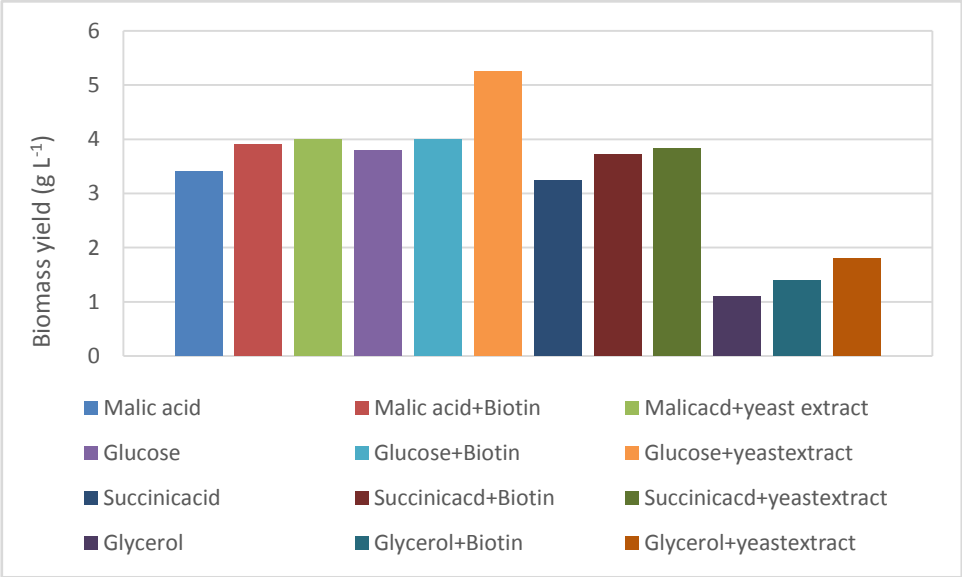

Fig 2. Biomass production by *A. lipoferum* Az204 in different carbon sources and carbon sources supplemented with biotin (0.1%) or yeast extract (0.1%) at 24 h of inoculation.

Glucose and malic acid were found to be the most suitable carbon sources for the growth of *A. lipoferum* Az204 although cell growth in glucose was comparatively higher. Glucose as carbon source produced 8.2 log cfu ml-1 number of cells at the end of 24 h and addition of biotin and yeast extract further enhanced cell growth to 8.65 log cfu ml-1 and 11.8 log cfu ml-1 respectively. Addition of yeast extract to glucose was found effective in increasing the cell growth. Glucose was followed by malic acid which produced 8.02 log cfu ml-1 of cells when used separately and cell number increased to 8.43 log cfu ml-1 and 9.25 log cfu ml-1 with the addition of biotin and yeast extract respectively. Growth of *A. lipoferum* Az204 was lesser in succinic acid when compared to glucose and malic acid. Succinic acid alone produced 7.82 log cfu ml-1 number of cells at the end of 24 h addition of biotin and yeast extract caused increase in cell number to 8.02 log cfu ml-1 and 8.22 log cfu ml-1 respectively. Glycerol showed lesser growth, when used alone it showed 4.37 log cfu ml-1 when used along with biotin and yeast extract showed the cell number of 4.5 log cfu ml-1 and 5.5 log cfu ml-1 respectively.

3.1 Effect of different carbon sources on the biomass production

The effect of different carbon sources on biomass production of *A. lipoferum* Az204 is shown in Figure 2. The highest cell biomass of 5.25 g L-1 was achieved by using glucose as a carbon source in combination with yeast extract. The effectiveness of glycerol in biomass productivity was less than other carbon sources. Very low microbial biomass growth was recorded when carbon source without any growth promoting nutrient was used for cultivation. The contribution of biotin and yeast extract in increase in biomass over respective carbon source was given in Table.1

Table 1. Biomass production by *A. lipoferum* Az 204 in Nfb broth at 24 h and percent increase in biomass due to biotin or yeast extract

Carbon sources	Biomass g L^{-1}			Increase in biomass (%)	
	Carbon source alone	Carbon source + biotin	Carbon source + yeast extract	With addition of biotin	With addition of yeast extract
Malic acid	3.42	3.91	4.13	14.71	17.65
Succinic acid	3.25	3.72	3.82	14.46	17.54
Glycerol	1.17	1.43	1.82	27.27	63.63
Glucose	3.81	4.24	5.25	5.26	38.16

3.2 Changes in pH during the growth of *A. lipoferum* Az204 in different carbon sources.

The time course changes in pH during the growth of *A. lipoferum* Az204 are presented in Table 2.Initial pH was adjusted to 6.8 and increase in pH was recorded during *A. lipoferum* Az204 growth upto 24 h. *A. lipoferum* Az204 grown in glucose + yeast extract reduced the pH from 6.8 to 6.45±0.05, while in glycerol and yeast extract combination pH was reduced from 6.8 to 6.5 ±0.01. In malic acid + yeast extract the pH increased from 6.8 to 7.71 ±0.03 and in succinic acid pH increased from 6.8 to 7.6±0.04. Malic acid and succinic acid when used separately increased the pH to 7.5±0.01 and 7.3±0.05 respectively but when used with biotin increase in pH to 7.57 ±0.02and 7.34±0.01 was recorded in malic acid and succinic acid respectively. Glucose and glycerol when used separately decreased the pH to 6.51±0.06 and 6.64±0.02 respectively but when used with biotin reduction in pH to 6.49±0.02and 6.61±0.01was recorded.

Table 2. Time course changes in pH during growth of *A. lipoferum* Az204 in Nfb medium containing different carbon sources

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Time (h)	pH											
	Malic acid	**Succinic acid**	**Glycerol**	**Glucose**	**Malic acid + biotin**	**Succinic acid + biotin**	**Glycerol + biotin**	**Glucose + biotin**	**Malic acid + yeast extract**	**Succinic acid + yeast extract**	**Glycerol + yeast extract**	**Glucose + yeast extract**
0	6.80 ±0.02	6.80±0.03	6.80±0.04	6.80±0.02	6.80 ±0.02	6.80 ±0.02	6.80 ±0.01	6.80 ±0.02	6.80 ±0.02	6.80 ±0.04	6.80 ±0.02	6.80 ±0.02
2	6.82 ±0.02	6.81±0.02	6.8±0.03	6.79±0.04	6.83 ±0.01	6.81 ±0.03	6.79 ±0.01	6.79 ±0.03	6.83 ±0.02	6.82 ±0.01	6.79 ±0.03	6.78 ±0.03
4	6.85 ±0.01	6.82±0.04	6.79±0.02	6.77±0.03	6.86 ±0.01	6.83±0.04	6.78 ±0.02	6.75 ±0.04	6.9 ±0.02	6.83 ±0.05	6.77 ±0.04	6.74 ±0.01
6	6.87 ±0.01	6.85±0.05	6.78±0.03	6.72±0.02	6.89 ±0.03	6.86 ±0.02	6.76 ±0.06	6.7 ±0.02	6.92 ±0.04	6.89 ±0.02	6.76 ±0.06	6.68 ±0.01
8	7.01 ±0.01	6.88±0.02	6.76±0.07	6.62±0.03	7.03±0.04	7.00 ±0.01	6.74 ±0.04	6.59 ±0.02	7.31 ±0.03	7.05 ±0.03	6.7±0.02	6.53 ±0.02
10	7.15 ±0.04	7.03±0.07	6.74±0.02	6.57±0.05	7.18 ±0.01	7.12 ±0.04	6.7 ±0.04	6.54 ±0.04	7.35 ±0.02	7.2 ±0.03	6.65 ±0.02	6.52 ±0.04
12	7.3 ±0.05	7.12±0.02	6.68±0.05	6.55±0.07	7.32 ±0.07	7.26 ±0.02	6.65 ±0.02	6.53 ±0.03	7.52 ±001	7.4 ±0.04	6.59 ±0.04	6.5 ±0.04
24	7.5 ±0.01	7.3±0.05	6.64±0.02	6.51±0.06	7.57 ±0.02	7.34 ±0.01	6.61 ±0.01	6.49 ±0.02	7.71 ±0.03	7.6 ±0.04	6.5 ±0.01	6.45 ±0.05

Data are Mean ± Standard error. Mean values differ significantly at $p < 0.05$.

4. DISCUSSION

The diazotrophic plant-associated bacterium *Azospirillum spp*., which resides in the rhizosphere, was rediscovered in the 1970s during studies using a semi-solid, buffer-free, nitrogen-free medium (NFb). This medium was primarily based on organic acids, particularly malate and succinate, which are the preferred carbon sources for this bacterium in its natural habitat (Day and Döbereiner.,1976). However, this medium yielded small populations and required improvement for bioinoculant production. From the outset, research on the interaction between *Azospirillum* and plants consistently highlighted the bacterium's strong affinity for organic acids, especially malate, as carbon sources [11]. Organic acids like malic acid and succinic acid are commonly found in the root exudates of many plants [12]. Malic acid, in particular, was initially incorporated into the traditional growth media for this species, as mentioned earlier. Subsequent physiological studies revealed that *Azospirillum* is highly adaptable to meet its carbon requirements (Hartmann and Zimmer *et al*., 1994). Glycerol is a common by-product of the transesterification of lipids and fats and had been previously evaluated for the growth of *Bradyrhizobium* used in inoculants [13]. It's important to note that the *Azospirillum* species used in this study is *A. lipoferum*, which prefers glucose as a carbon source. However, glucose is not used by some *Azospirillum* species, such as the commonly used inoculant *A. brasilense*, and is not their

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preferred carbon source within this genus [14]. In this study the evolution of pH depended upon the culture media used. The pH variations observed in the cultures reflect the metabolic activity of the bacterium and the utilization of different carbon sources. Overall, the pH trends suggest that malic acid and succinic acid cultures tend to show slight increase pH throughout the growth period. In contrast, cultures with glucose and glycerol exhibit a gradual decrease in pH, which may be attributed to the production of organic acids during bacterial growth. When biotin is introduced, it appears to have a minimal impact on pH regulation, as the pH trends remain consistent with those of cultures without biotin. However, the addition of yeast extract alongside carbon sources shows a distinct pH pattern Specifically, cultures with glucose and yeast extract exhibit a sharp decrease in pH, indicating increased metabolic activity and possible organic acid production. These results highlight the complex interplay between carbon sources, growth-promoting nutrients, and pH regulation in *A. lipoferum* Az204 cultures. The production of acidic or alkaline metabolic products by organisms growing in media at varying pH may be another important means of pH regulation [15].To enhance growth and reduce lag phase, the carbon sources were supplemented with biotin or a small amount of yeast extract. In the present investigation with *A. lipoferum* Az204 the preference of the strain for glucose and malic acid was the same. The organism exhibited better growth in terms of viable cell number and biomass in the presence of yeast extract rather than biotin. The combination of glucose and yeast extract outperformed all other combinations. Similar effect of Yeast Extract was observed in *Rhodobacter sphaeroides* where the effect of yeast extract as nitrogen source for promotin cell growth and boosting hydrogen production in *R. sphaeroides* strains MDC6521 and MDC6522, which were isolated from mineral springs of Armenia [16]. During growth it was found that yeast extract, at a concentration of 2 g L^{-1} proved to be a highly effective nitrogen source, leding to increased bacterial cell growth. *Rhizobium sp* where a low concentration of yeast extract (0·1%) in liquid media favoured rapid growth and high percentage of viable cells in cultures of *Rhizobium japonicum* (CB 1809), *R. lupini* (WU 425), *R. meliloti* (SU 47), *R. trifolii* (TA1) and a cowpea strain (CB 756) [17].

5. CONCLUSION

The effect of various carbon sources on viable cell number and biomass yield of the plant growth promoting bacterial bioinoculant *A. lipoferum* Az204 with and without growth promoting nutrients in the gowth medium was investigated. Among all the carbon sources studied glucose supported high viable cell number of *A. lipoferum* Az204 and addition of yeast extract further enhanced the cell growth and increased the biomass. Hence it was found that the modified Nfb medium with glucose as carbon source and supplemented with 0.1% of yeast extract is economical for the commercial production of this strain.

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