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Indole-3-acetic acid production by *Streptomyces albidoflavus*

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A terrestrial streptomycete isolate, *Streptomyces albidoflavus* was screened for plant growth promoters like indole-3-acetic acid (IAA). *Streptomyces albidoflavus* was found to produce IAA in culture medium supplemented with L-tryptophan. IAA production by the strain initiated after 24 hrs and reached a maximum after 96 hrs of incubation when it reached stationary phase of growth. IAA was extracted with ethyl acetate from a culture filtrate (96 hrs) and it was purified and structurally confirmed by ^{1}H NMR, ^{13}C NMR and EI-MS. Cultural and nutritional conditions were optimized for IAA production by this strain. The strain produces high amounts of IAA when cultured in a medium containing glucose (1%) and yeast extract (0.5%) as carbon and nitrogen sources respectively, along with L-tryptophan (0.5%). The optimum pH and temperature for IAA production were 7 (at 30°C) and 35°C (at pH = 7), respectively. The strain, *S. albidoflavus* might be useful for plant growth promoters like IAA beside antimicrobial metabolites.

Key words: *Streptomyces albidoflavus*, IAA, production, identification.

INTRODUCTION

The production of indole-3-acetic acid (IAA) is widespread among fungi and bacteria (Gruen, 1959; Loper & Schroth, 1986). IAA formation is believed to be a major property of the rhizosphere and the epiphytic and symbiotic bacteria that stimulate and facilitate plant growth (Wichner & Libbert, 1968). On the other hand, certain free-living microorganisms (i.e. those that develop no association with plants in the course of their life cycle) are also capable of synthesizing phytohormones (Tien *et al.,* 1979). Generally, IAA biosynthesis *via* indole-3-acetamide takes place in certain streptomycetes and phytopathogenic pseudomonads and xanthomonads. In this pathway, tryptophan is converted to indole-3-acetamide (IAM) by tryptophan-2-monooxigenase and IAM is metabolized to IAA by IAM-hydrolase (Fett *et al.*, 1987; Manulis *et al.,* 1994; Matsukawa *et al.,* 2007). Most *Streptomyces* spp. are useful as biocontrol agents and elaborate bioactive metabolites (Berdy, 2005). During the screening of actinomycetes for bioactive metabolites, a *Streptomyces* sp. was found to produce indole compounds. Taxonomic study on this strain showed that it is related to the *Streptomyces albidoflavus* cluster (Narayana *et al.,* 2007). In the present report, an attempt was made to investigate the production of IAA from *Streptomyces albidoflavus*.

MATERIALS AND METHODS

Growth condition and IAA production

The strain *S. albidoflavus* was maintained on yeast extract – malt extract – dextrose agar (YMD) medium (0.4% yeast extract, 1% malt extract, 0.4% dextrose, 0.2% K_2HPO_4, 2% Agar, pH = 7) (Li, 1997).

A culture suspension of the strain was inoculated in YMD broth supplemented with 0.05% L-trypto-

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phan at 30°C and pH 7.0. The effects of the incubation period on the production of IAA by the strain were studied. Cell dry weight was also studied for biomass production to evaluate the relationship between cell growth and IAA production. Cell dry weight was determined by filtering the culture medium through pre-weighed Whatman filter paper No. 44. Filter papers contained biomass were placed in an oven at 80°C for 18 hrs before being weighed again (Corvini *et al.,* 2000). The amount of IAA produced in the culture broth was quantified by using the colorimetric assay suggested by Glickmann & Dessaux (1995).

Extraction of IAA

After 96 hrs of cultivation, fermentation of the culture broth was stopped. The culture filtrate was collected and adjusted to pH 3.5 with 1N HCl. The acidified culture filtrate was then extracted with ethyl acetate and vacuum dried at 37°C. IAA was partially purified from the crude solvent extract by using silica gel column chromatography (22×5 cm) and fractions were collected with the solvent system of ethyl acetate and hexane (20:80 v/v). Each fraction was tested on TLC with the solvent system and then developed with the Salkowski reagent (0.01M $FeCl_2$ in 35% $HClO_4$) (Ehmann, 1977). The indole containing fraction was further purified in preparative high-performance liquid chromatography (HPLC, Shimadzu, Japan) (normal phase, silica column, 10×250 mm, 5 μl using hexane/2-propanol, 8:2) and the pure compound obtained was structurally identified by proton nuclear magnetic resonance spectroscopy (^{1}H NMR), ^{13}C NMR and Electron Ionization Mass Spectra (EI-MS) (Fotso *et al.*, 2003). Both ^{1}H- and ^{13}C-NMR spectra were obtained in CD_3OD on a Bruker DRX-500 NMR spectrometer operating at 300 MHz. EI-MS were taken on a Shimadzu QP5000 mass spectrometer.

Optimization of IAA production

The optimum concentration of the precursor L-tryptophan for IAA was studied by adding different concentrations of L-tryptophan to the basic medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.2% $CaCO_3$) supplemented with 1% D-glucose as carbon source (Majumdar & Majumdar, 1965). The impact of various carbon (D-glucose, maltose, mannitol, galactose, fructose, sucrose, starch, lactose and trehalose) and nitrogen (KNO_3, $NaNO_3$, $(NH_4)_2SO_4$, L-asparagine, L-glutamine, L-tyrosine, yeast extract, peptone, soybean meal) sources on IAA production was investigated by using the basic medium with an optimum level of L-tryptophan. Carbon compounds were added in 1% concentration to the basic medium in replacement of D-glucose.

The effects of various nitrogen sources on IAA production were studied by adding the nitrogen source (0.2%) to the basic medium replacing 0.2% $NaNO_3$, with D-glucose (1%) used as carbon source. The pH of the respective medium was adjusted to 7.0. The optimal concentrations of the best carbon and nitrogen sources were also determined for maximum production of IAA. The effects of pH and temperature on IAA production were studied. The pH of culture medium was adjusted to different values ranging from 5 to 9 before introducing the inoculum into the L-tryptophan supplemented YMD broth. The inoculated culture medium was incubated at 30°C. The impact of temperature on IAA production was determined by incubating the culture medium (with pH 7) at different levels of temperature (20-45°C, at 5°C intervals).

Statistical analysis

Data regarding IAA production by *S. albidoflavus* was statistically analyzed with t-test using SigmaPlot 11.0 (Systat Software Inc., USA).

RESULTS AND DISCUSSION

The *S. albidoflavus* strain started IAA production after 24 hrs of culturing and reached a maximum after 96 hrs. The amount of biomass as well as IAA production increased simultaneously. Maximum production of IAA was achieved during the stationary phase of the culture (Fig. 1). The effect of incubation period on IAA was statistically significant (t-test, $p<0.05$). Cacciari *et al.* (1989) have reported accumulation of IAA from *Azospirillum* and *Arthrobacter* sp. during the stationary phase.

The pure compound of indole obtained during the HPLC purification step was structurally confirmed by ^{1}H NMR, ^{13}C NMR and EI-MS as IAA. The ^{1}H NMR spectrum of the compound in CD_3OD at 300 MHz showed signals at 3.70δ (sharp, S, 2H), 7.0δ (t, aromatic-C-H), 7.10δ (t, aromatic-C-H), 7.15 δ (S,-C-H, broad), 7.32δ (d, aromatic-C-H), and 7.51δ (d, aromatic-C-H). The ^{13}C NMR spectrum of IAA in CD_3OD at 300 MHz disclosed 10 carbon atoms and exhibited signals at 30.59 (s, C-10), 107.48 (s, C-3), 110.84 (s, C-7), 118.05 (s, C-6), 118.42 (s, C-2), 121.04

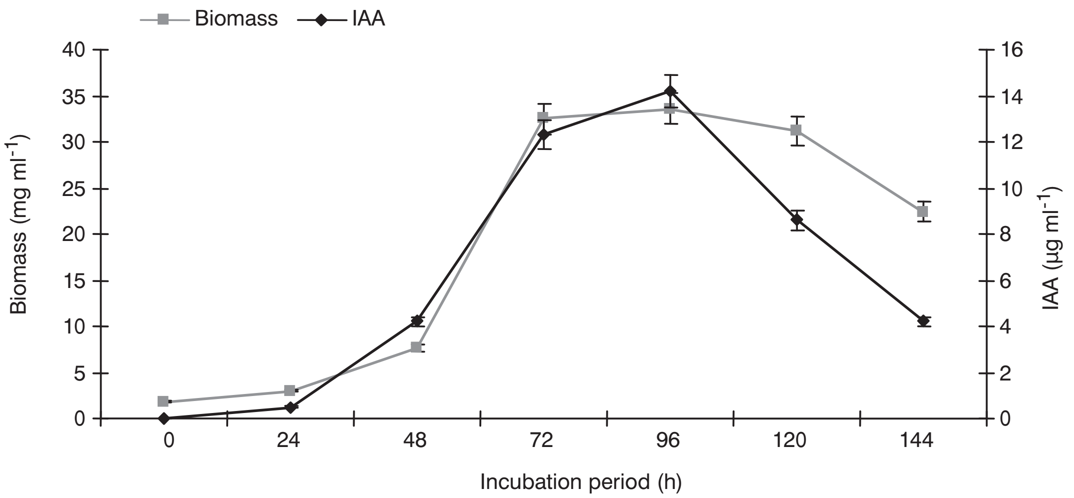

FIG. 1. Effect of incubation period on growth and IAA production (mean values of three replicates, bars represent ±SD) (t-test, $p < 0.05$).

(s, C-4), 123.22 (s, C-5), 127.27 (s, C-8), 136.61 (s, C-9) and 175.06 (s, C-11). The mass spectrum (EI-MS) of the compound displayed *m*/z at 176 (+M), 130 and 198 suggesting a molecular weight of 176. Based on the above data, the pure compound from the crude extract of the strain was structurally confirmed as indole-3-acetic acid.

L-tryptophan is generally considered as an IAA precursor, because its addition to IAA producing bacterial culture enhances IAA biosynthesis (Costacurta & Vanderleyden, 1995). The strain *S. albidoflavus* preferred tryptophan for production of IAA. Maximum IAA production was found in the medium amended with 0.5% tryptophan (Fig. 2). Tryptophan at levels above 0.5% resulted in a decline of IAA production. IAA production was not observed in the L-tryptophan free medium. There was a significant difference between the amounts of IAA produced at different levels of L-tryptophan (t-test, $p < 0.001$). For many bacteria, the conversion of tryptophan into IAA is of utmost importance (Tsavkelova *et al.,* 2006). Manulis *et al.* (1994) have reported that various *Streptomyces* spp. including *S. violaceus*, *S. scabies*, *S. griseus*, *S. exfoliatus*, *S. coelicolor* and *S. lividans*, secrete indole-3-acetic acid (IAA) when fed with tryptophan. They also stated that the omission of tryptophan from the culture medium decreases the level of IAA synthesis by the microorganisms. The strain *S. albid-*

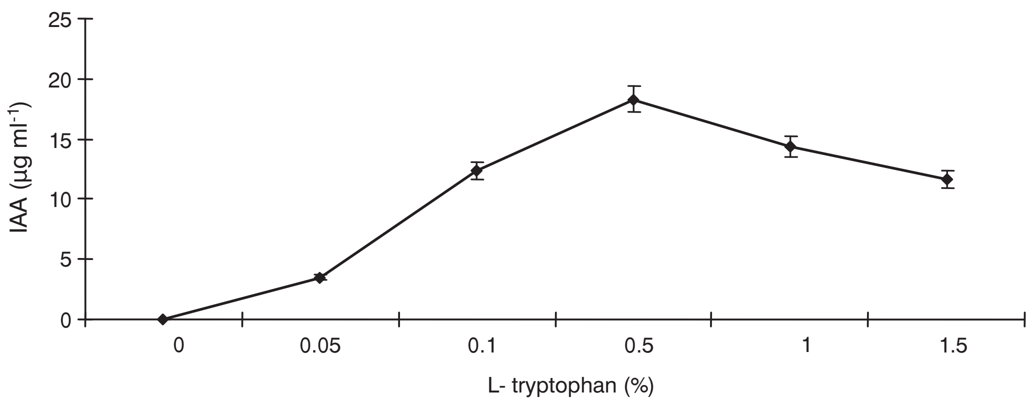

FIG. 2. Optimum concentration of L-tryptophan for IAA production (mean values of three replicates, bars represent ±SD) (t-test, $p < 0.001$).

TABLE 1. Effects of different carbon sources on IAA production by *S. albidoflavus*

Carbon source (1%)	IAA (μg ml^{-1}) mean values ± SD
Control*	2.4 ± 0.35
D-glucose	26.5 ± 1.62
Maltose	24.4 ± 0.49
Mannitol	12.3 ± 1.48
Galactose	10.2 ± 0.91
Fructose	3.7 ± 0.49
Sucrose	2.6 ± 0.07
Starch	16.6 ± 1.62
Lactose	3.2 ± 0.70
Trehalose	14.5 ± 0.49

* Control = Culture medium without any additional carbon source (with 0.5% L-tryptophan)

TABLE 2. Effects of different nitrogen sources on IAA production by *S. albidoflavus*

Nitrogen source (0.1%)	IAA (μg ml^{-1}) mean values ± SD
Control*	7.4 ± 1.41
KNO_3	10.2 ± 0.35
$NaNO_3$	12.4 ± 0.56
$(NH_4)_2SO_4$	6.9 ± 0.42
L-Asparagine	12.2 ± 1.27
L-Glutamine	11.2 ± 0.98
L-Tyrosine	3.7 ± 0.34
Yeast extract	29.2 ± 1.06
Peptone	13.5 ± 0.91
Soybean meal	18.9 ± 0.52

* Control = Culture medium without any additional nitrogen source (with 0.5% tryptophan and 1% D-glucose)

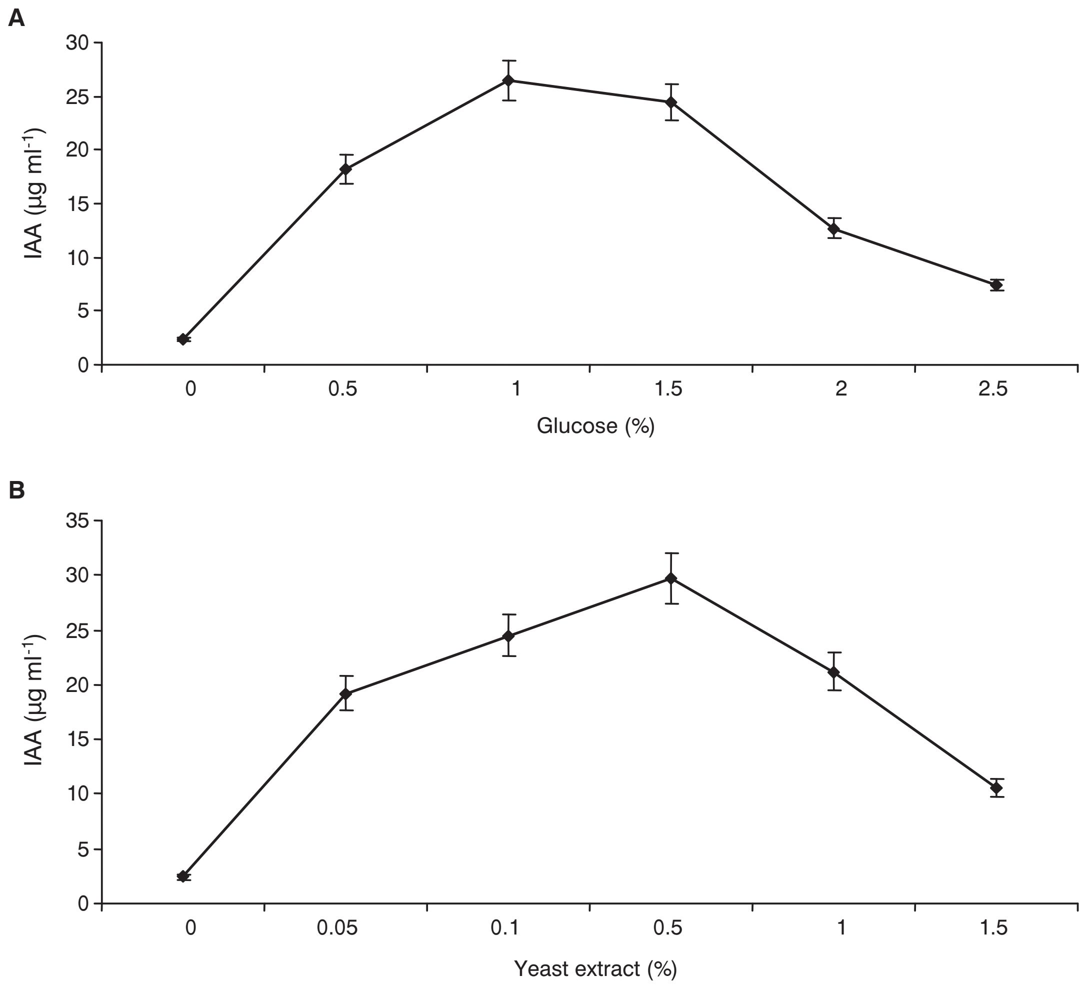

FIG. 3. Optimum levels of glucose (A) and yeast extract (B) on IAA production (mean values of three replicates, bars represent ±SD) (t-test, $p < 0.001$).

oflavus was also found to elaborate maximum levels of IAA in a medium containing an optimum amount (0.5%) of tryptophan.

The impact of different carbon sources on IAA production was tested on IAA production (Table 1). The most suitable carbon source for IAA production was glucose, followed by maltose, starch, trehalose, mannitol and galactose. The impact of carbon sources on IAA production was statistically significant (t-test, $p < 0.05$). Among the inorganic nitrogen sources, $NaNO_3$ was found to be the most suitable nitrogen source for IAA production (Table 2). Organic nitrogen sources promoted IAA production than inorganic nitrogen sources. Maximum IAA production was observed in the medium containing yeast extract as nitrogen source. There was a significant difference between the concentrations of IAA influenced by nitrogen (t-test, $p < 0.001$). High levels of IAA production were achieved when the medium was supplemented with glucose (1%) and yeast extract (0.5%) as carbon and nitrogen sources, respectively (Fig. 3). Basu & Ghosh (2001) have reported that glucose and KNO_3 are the best carbon and nitrogen sources for IAA production by *Rhizobium* spp. Shilts *et al.* (2005) have reported high IAA production by *Colletotrichum acutatum* in medium containing mannitol (as carbon source) and ammonium nitrate (as nitrogen source) in addition to tryptophan.

The impact of pH on IAA production by the specific *S. albidoflavus* strain was determined by adjusting the culture medium to different levels of pH (5-9) and incubated at 30˚C. Maximum amount of IAA

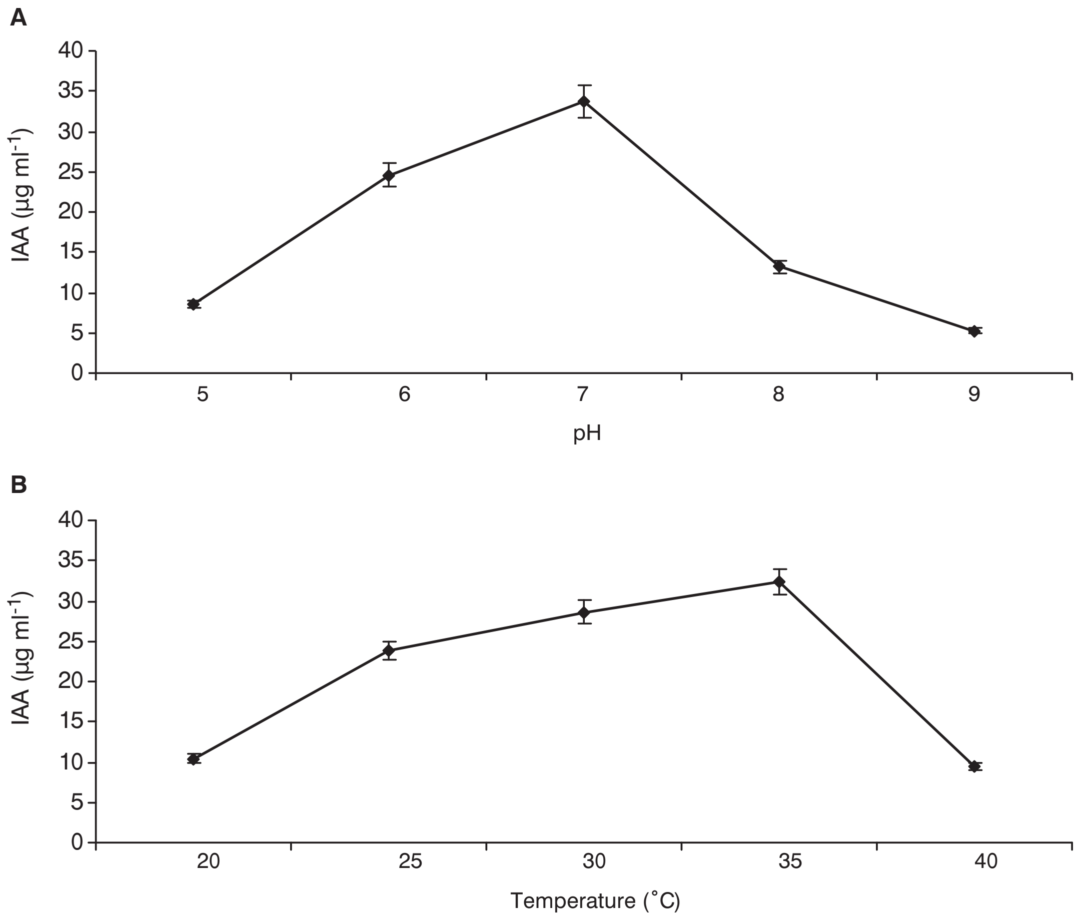

FIG. 4. Impact of pH (A) and temperature (B) on IAA production (mean values of three replicates, bars represent ±SD) (t-test, $p < 0.001$).

was produced when pH of the culture medium was set to 7. Acidic pH (below 6) and alkaline pH (above 8) were found to be unfavorable for IAA production by this strain. The effect of temperature on IAA production by this strain was studied by incubating the culture medium (pH 7) at different ranges of temperature (20-40 °C). The optimum temperature for IAA production was 35 °C. The amounts of IAA decreased as temperature dropped below 25 °C and at temperature higher than 40 °C. The strain elaborated maximum IAA production when the medium was adjusted to pH 7 and grown at 35 °C (Fig. 4). IAA production by the strain was statistically different at different pH and temperature values (t-test, $p < 0.001$). Mandal *et al.* (2007) have reported that the *Rhizobium* strain VMA 301 elaborated high levels of IAA production in medium containig glucose, KNO_3 and L-tryptophan at pH 7.2.

Matsukawa *et al.* (2007) have reported that *Streptomyces* spp. such as *S. purpurascens*, *S. coelicolor*, *S. olivaceus*, and *S. kasugaensis* produced IAA at concentrations of 28.4, 21.8, 14.2 and 51.5 μg ml^{-1}, respectively (under optimum culture conditions). The *S. albidoflavus* strain under investigation produced an amount of 34 μg ml^{-1} of IAA at optimal culture conditions. Secondary metabolites produced by this strain were found to have an inhibitory effect on plant pathogenic fungi (Narayana *et al.,* 2007). Microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells (Kravchenko *et al.,* 2004). The application of organic fertilizers can increase the levels of tryptophan in soil and tryptophan found in organic wastes and fertilizers may be produced by aerobic or anaerobic microbial transformation (Arkhipchenko *et al.,* 2006). Soil microorganisms can utilize natural source of tryptophan and elaborate plant growth promoters like IAA. The present study might be useful to establish *Streptomyces albidoflavus* for plant growth promoters like auxins in addition to its antimicrobial properties and it would be useful to convert natural tryptophan source to IAA.

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