

Original Research Article

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Isolation and Characterization of Nicotine Reducing Probiotics

Bhavika Chauhan and Nileshkumar Pandya*

Department of Microbiology, Arts, Science & Commerce College, Kamrej Cross Road, Surat,
Gujarat-394185, India

*Corresponding author

ABSTRACT

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A total of seven microorganisms were isolated from different probiotic sources. The nicotine reducing activity of isolates was tested by general analytical techniques. Amongst them isolates BC6 and BC7 possess maximum nicotine reducing activity therefore it was selected for further investigation. Both isolates could utilize up to 1 g/l nicotine as the sole source of carbon and nitrogen, and optimum nicotine reducing activity obtained at pH 7 by inoculating with inoculum size of 1 McFarland standard units. After morphological and biochemical characterization and according to information given on leaflet of probiotic product, isolate BC6 and BC7 were identified as *Lactobacillus sporogenes* and *Lactobacillus casei*, respectively.

Introduction

Nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine is an alkaloid found in the nightshade *Solanaceae* family of plants, predominantly in the leaves of tobacco and it is a colorless, less to pale yellow, hygroscopic oily liquid (Hossain and Salehuddin, 2013). People can smoke, chew or sniff tobacco. Smoked tobacco products include cigarettes, cigars, bidis and kretek and chewed tobacco products include chewing tobacco, snuff, dip and snus; snuff can also be sniffed (NIDA, 2018). Nicotine is an addictive substance that can lead to nicotine dependence and addictive behaviour in human populations (Niu *et al.*,

2000). Nicotine can cause cancer, gene mutation & malformation (Yildiz, 2004) and neurotoxin developmental effects of nicotine can naturally affect a variety of cellular processes such as generation of oxidative radicals, apoptosis and hyperplasia of cell, enhancing gene expression to secrete hormones and regulation of enzymatic activity (Yildiz, 2004; Yildiz *et al.*, 1998; Qiao *et al.*, 2005 and Gurusamy and Natarajan, 2013).

A number of microorganisms able to degrade nicotine have been isolated and characterized till now, including bacteria, actinomycetes and fungi (Brandsch, 2006; Li *et al.*, 2010; Meng *et al.*, 2010; Wang *et al.*, 2009 & Li *et al.*,

2014). No one is isolated and characterized probiotic for removal of nicotine on human beings yet. Tobacco waste degrading bacteria can't directly use on human as it may be adversely affect human beings. The intention of this study is to isolate and characterise probiotics that can reduce nicotine concentration in the human body.

Materials and Methods

Isolation of probiotics

For isolation of probiotics, various probiotic sources such as Curd, Vitagut probiotic capsule and Yakult fermented milk drink were streaked on sterile nutrient agar plate and sterile MRS agar plate and incubated at 37 °C for 48 hrs. After incubation developed colonies were transferred and streaked on the new agar plates repeatedly until a pure isolate was obtained. Isolates were transferred individually on sterile nutrient agar slant and preserved at 4 °C for further use.

Enrichment culture and isolation and identification of nicotine reducing probiotics

Probiotics isolated as above were enriched for determining their nicotine reducing activity. The isolates were added into sterile liquid nicotine medium (NIM) containing Na₂HPO₄ 6 g/l, KH₂PO₄ 3 g/l, NH₄Cl 1 g/l, NaCl 0.5 g/l, MgSO₄ 0.12 g/l, CaCl₂ 0.1 g/l and nicotine 0.2 g/l, (Wei *et al.*, 2008) in which nicotine (extracted from tobacco; Singh *et al.*, 2013) was the sole source of carbon and nitrogen, nicotine was sterilized separately by filtration and added into media before inoculation and broth were inoculated with isolates and incubated at 37 °C. After 4 days, the culture was used as inoculum and streaked onto the solid nicotine medium plates containing 0.2 g/l nicotine. After incubation at 37 °C for 72 hrs, colonies began to appear on the plates and these isolates were selected for this study.

Selected isolates were morphologically and biochemically characterized by Gram's staining and various biochemical test as per standard methods (Cappuccino and Sherman, 1992).

Screening of microorganisms for nicotine reducing activity

Different isolates obtained on NIM agar plates were screened for their nicotine reducing activity on sterile NIM broth containing 0.5 g/l nicotine. Isolates were inoculated in sterile NIM broth and incubated at 37 °C and determined cell density by taking absorbance at 600nm (Liu *et al.*, 2014) and nicotine reducing activity at 1 hr intervals. Un-inoculated NIM broth containing 0.5 g/l nicotine was used as control.

Analytical method for determining nicotine reducing activity of isolates

Spectrophotometric method

At predetermined time intervals, the samples of inoculated broth were taken and cells were removed by centrifugation at 10,000 rpm for 10 min. 1 ml of the supernatant was mixed with 0.5 ml of bromine water and shaken it for 2 min. By the drop wise addition of formic acid, the excess of bromine was removed then 1 ml each of potassium iodide and leuco crystal violet was added and shaken for few minutes. 1 ml of 2 M sodium hydroxide was added drop wise and made final volume to the 25 ml with double distilled water and the absorbance was measured at 592 nm after 25 min (Asthana *et al.*, 2004).

Thin Layer Chromatography (TLC)

After 6 hours of nicotine broth inoculation with isolates, the samples were centrifuged and supernatant was used for TLC analysis. On silica gel 60 F₂₅₄ TLC aluminium sheets, nicotine reducing activity of microbes were

determined by using Toluene – Isopropanol – Ammonia (100:25:1) as a mobile phase and spraying with Dragendroff's reagent (Svendsen *et al.*, 2011).

Optimal conditions for growth and nicotine reducing activity of isolates

Potent microbes were selected by primary screening and its maximum nicotine reducing activity was determined by inoculating them in sterile NIM broth containing nicotine concentration ranging from 0.5 to 1.5 g/l and determined cell density and nicotine reducing activity as predetermined methods. Un-inoculated nicotine medium were used as control. The effect of pH on isolates was tested by adjusting a variety of pH condition of broth with 1M of HCl or NaOH to pH 6 to 8, respectively. For determining the effect of inoculum size on nicotine reducing activity, the nicotine broth was inoculated with various inoculum sizes adjusted to 0.5, 1.0 and 2.0 McFarland Standard units and analysed their nicotine reducing activity (McFarland J., 1907). All experiments were performed in triplicate.

Results and Discussion

Isolation of probiotics

Total 8 isolates were obtained on Nutrient agar and MRS agar plates, amongst them 4, 3 and 1 from curd, vitagut capsule and yakult fermented milk drink, respectively.

Enrichment culture and isolation and identification of nicotine reducing probiotics

After 4 days of Isolate addition into liquid nicotine medium, turbidity observed in broth indicating their growth and their colony formation on NIM agar plate containing

nicotine as sole source of carbon and nitrogen after 72 hrs indicating they possessed nicotine reducing activity.

All isolates able to form visible colony on the nicotine agar plate except one isolated from curd showing that it was incompetent to reduce nicotine concentration.

The colony morphology of isolate BC6 was large, round, moist, opaque, raised and entire edge. It was a Gram-positive and long rod shaped bacterium. The colony morphology of isolate BC7 was large, round, mucoid, opaque, flat & entire edge. It was a Gram-positive and short rod shaped bacterium. Isolate BC6 and BC7 showing maximum nicotine reducing activity were selected for this investigation, which were identified as *Lactobacillus sporogenes* and *Lactobacillus casei* and obtained from vitagut capsule and yakult fermented milk drink, respectively. They are identified from morphological and biochemical characterization and according to information given on leaflet of probiotic product.

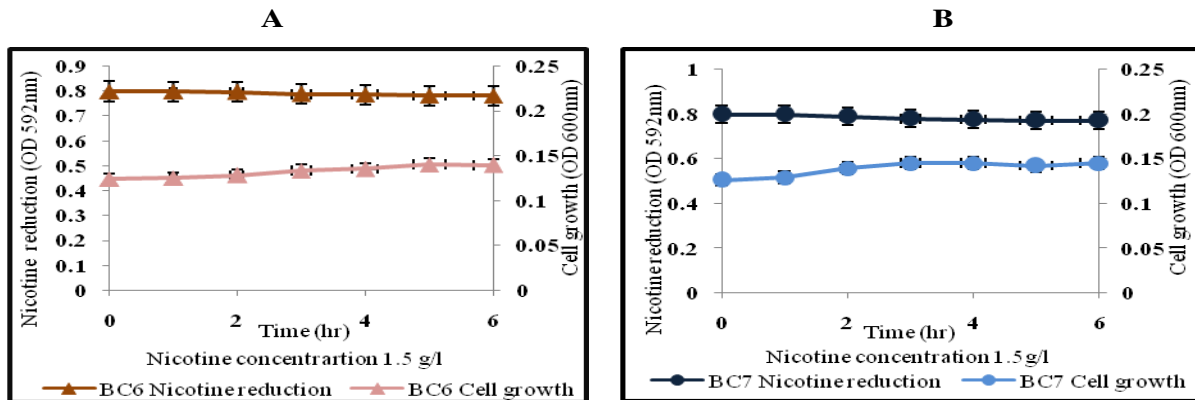
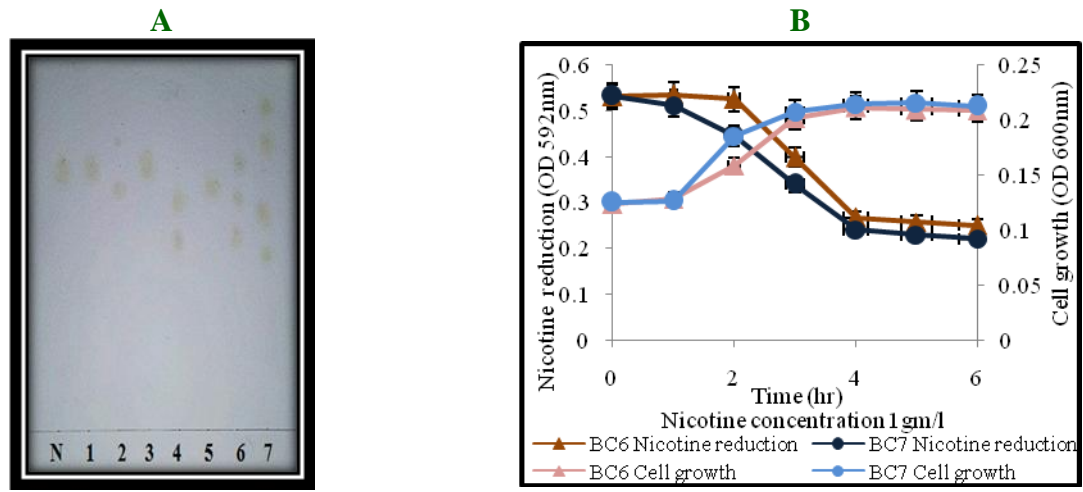
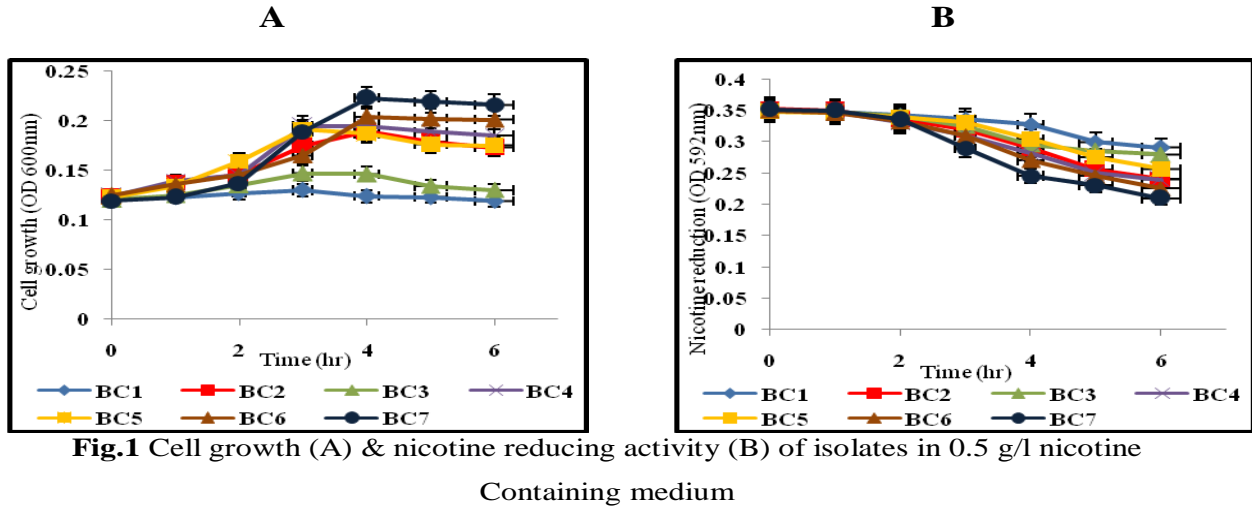
Screening of microorganisms for nicotine reducing activity

A seven isolates showing colony formation on the NIM agar plate and their nicotine reducing activity into NIM broth containing 0.5 g/l nicotine was determined by two methods.

Analytical method for determining nicotine reducing activity of isolates

Spectrophotometric method

The cell growth and nicotine reducing activity of isolates into nicotine medium was determined by taking optical density of broth at 600 nm and at 592nm as above described method, respectively.



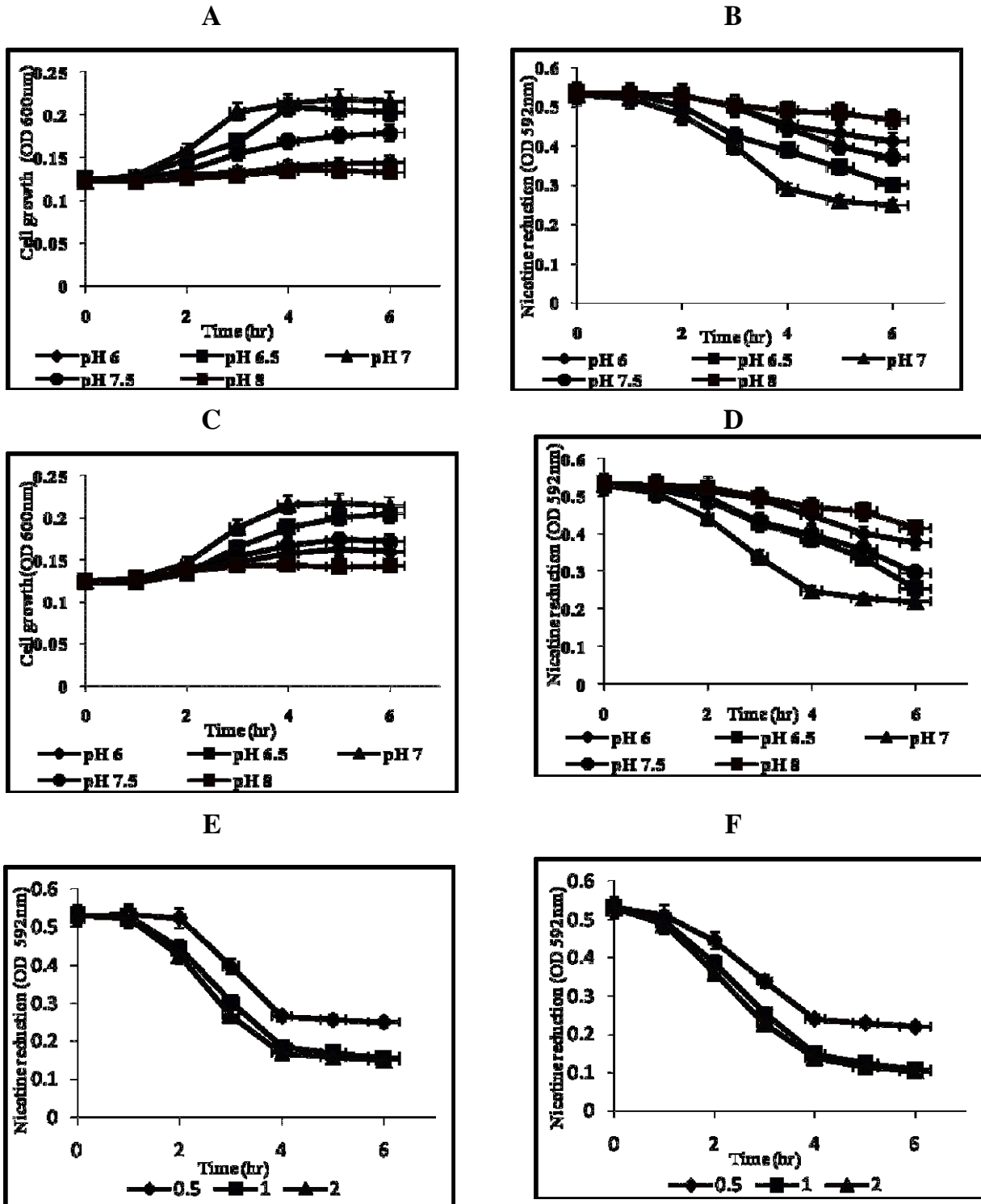


Fig.4 Effect of pH on cell growth of BC6 (A) & BC7 (C) and nicotine reducing activity of BC6 (B) & BC7 (D), Effect of inoculum size imicotine reducing activity of BC6(E) & BC7 (F)

As shown in figure 1(A), the growth of isolate BC6 and BC7 was observed maximum in compare to other. The depletion of absorbance of nicotine medium at 592 nm was suggested that nicotine concentration decreased in the broth.

A minimal optical density at 592 nm of nicotine medium inoculated with strain BC6 and BC7 after 6 hrs was manifested that their maximum nicotine reducing activity in compare to other isolates (Figure 1B).

Thin layer chromatography

After 6 hrs of incubation of isolates in 0.5 g/l nicotine containing medium, the result of TLC analysis were shown in figure 2(A) in which N indicated nicotine broth without microbes inoculation as control and nicotine broth inoculated with BC1, BC2, BC3, BC4, BC5, BC6 and BC7 were labelled as 1 to 7, respectively. In comparison to control, more spots observed with BC6 and BC7 indicates the probable nicotine destruction to more than one product.

Optimal conditions for growth and nicotine reducing activity of isolates

The effects of nicotine concentration on cell growth and nicotine reducing activity of two potent isolates were investigated at nicotine concentration ranging from 0.5-1.5 g/l. As shown in figure 2(B), the maximum cell growth and nicotine reducing activity of both isolates was observed at 1 g/l nicotine concentration and both isolates were unable to grow on 1.5 g/l nicotine containing medium so that nicotine reducing activity was not visible at this concentration. The obtained results are similar to the work done by Liu *et al.*, 2014, where the optimal growth of *Pseudomonas geniculate* N1 was observed at 1 g/l nicotine in which nicotine was the sole source of carbon and nitrogen.

A cell growth and nicotine reducing activity of isolates with nicotine was observed from pH 6 to 8. The maximum growth and nicotine reducing activity of both isolates on nicotine medium was at pH 7, which suggest that they prefers a neutral environment for reduction of nicotine concentration whereas the optimum growth of *Acinetobacter* sp. ND12 with nicotine was observed between pH 4 and pH 6 (Li *et al.*, 2011). In this study isolated bacteria showed optimal activity close to neutral pH and saliva pH also normally nearby it (Baliga *et al.*, 2013) so these strains may be helpful to reduce nicotine concentration from smokeless tobacco in mouth (Fig. 3).

The influence of inoculum size on cell growth and nicotine reducing activity were determined at different inoculum size ranged from 0.5 to 2 McFarland standard units. Figure 4(E) and 4(F) clearly indicated that the nicotine reducing activity of microorganisms improved as the inoculum size was increased. The nicotine broth inoculated with 2 McFarland standard unit inoculum size containing culture was shown the absorbance at 592 nm nearer to develop by 1 McFarland standard unit inoculum size containing culture of both isolates it suggest that the effect of inoculum size above 1 McFarland standard unit on nicotine reducing activity was become a stagnant.

Isolates BC6 and BC7 possess maximum nicotine reducing activity, they could utilize nicotine up to 1 g/l as the sole source of carbon and nitrogen. Optimum nicotine reducing activity obtained at pH 7 by inoculating the isolates with inoculum size of 1 McFarland standard units.

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