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Der Pharmacia Lettre, 2013, 5 (5):35-42
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ISSN 0975-5071
USA CODEN: DPLEB4

L-Tryptophan production by a psychrophilic *Pseudomonas* sp. 023K: A new report

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ABSTRACT

The present study was conducted to screen for psychrophilic bacterial isolate from soil samples of Kashmir that are able to produce excess L-tryptophan and accumulate it extracellularly. The morpho-physio-biochemical characters and 16S rDNA sequence homology of the isolated strain indicated its taxonomic affiliation to Pseudomonas chlororaphis. The isolated wild type Pseudomonas sp. was able to grow optimally at 10^0 C and as low as 0^0C and showed considerable amount of L-tryptophan production (119 mg/lit). Findings in this study indicate the possibility that the isolated psychrophilic strain may be used for industrial production of L-tryptophan. This is so far the first report on L-tryptophan production by any psychrophilic bacteria.

Keywords: Psychrophilic, *Pseudomonas* sp., L-tryptophan production, Thin Layer Chromatography.

INTRODUCTION

L-tryptophan is the third limiting essential amino acid required in the diet of protein deficient population and livestock after methionine [1] and lysine. In general cereal grains are almost lacking L-tryptophan . Recently L-tryptophan is used in large scale for the treatment of schizophrenia, [2], alcoholism, insomnia [3] and depression [4]. It is a safe and reasonably effective sleep aid, probably due to its ability to increase brain levels of serotonin [5]. The disorders, fructose mal-absorption and lactose intolerance [6] are caused due to improper absorption of tryptophan in the intestine and reduced levels of tryptophan in the blood. It is also the precursor of a number of plant metabolites like IAA [7], indigo, strychnine, serpentine, reserpine [8] and violacein [9]. A shift towards microbial processes for L-tryptophan production by microbial fermentation began in the early 1960s. Now a days L-tryptophan production is carried out through enzymatic [10] and fermentative processes using metabolically engineered microorganisms [11]. Pathway of tryptophan biosynthesis is very stringently regulated together with tyrosine and phenylalanine and also it is coded by a single codon UGG. In the last step of aromatic amino acid pathway, tryptophan synthase catalyzes the formation of tryptophan from indole and the amino acid serine. Although many reports are their on production of L-tryptophan from different mesophilic bacteria like *Escherichia coli* [12]; *Bacillus subtilis* [13]; *Aureobacterium flavescens* [11] and few thermophillic bacteria, *Pseudomonas hydrogenothermophila* [14]. No such reports are available on Psychrophilic bacteria till date. Primary aim of the present work is to screen and identify a psychrophilic bacterial strain that produce excess L-tryptophan.

MATERIALS AND METHODS

Isolation of psychrophilic bacterial strain

Soil samples were collected from Nalbandpora, Jammu and Kashmir, India, situated at 34.0897°N 74.7900°E in November 2010. 1 g of each soil sample were serially diluted separately up to 10^{-5} dilution and 0.1 ml of 10^{-2} and

10^{-3} dilution from each sample were spreaded on Davis and Mingioli [15] minimal agar medium containing KH_2PO_4 0.3g , K_2HPO_4 0.7g, Tri sodium citrate 0.05g, $MgSO_4.7H_2O$ 0.02g, $(NH_4)_2SO_4$ 0.1g, Glucose 1% , Agar 2 % **,** Distilled water 100ml. pH 7.2. The plates were incubated at 10^o C for 72-96 hrs. The bacterial isolates were sub-cultured on new Davis and Mingioli agar plate and purified by streaking twice

Screening of L-tryptophan producing bacterial strain
To screen a potent L-tryptophan producing bacterial strain all the isolates were grown in Davis and Mingioli medium. The amino acid produced extracellularly in the medium was identified by thin layer chromatography (TLC).

Among all the tested isolates the best L-tryptophan producing strain was selected for further studies.

Detection of L-tryptophan by TLC
The amino acid produced was identified by thin-layer chromatography on silica gel 60 F254 plates (MERCK) with a solvent system of n-butanol/acetic acid/water (65:13:22 V/V/V). The supernatant obtained by centrifugation of culture medium at 8000 rpm for 5 min from 72 hrs old culture was mixed well with n-butanol solvent (1:2). It was incubated at room temperature for 3hrs so that two phases were formed. After taking organic phase followed by evaporation, 50 µl of the concentrate was loaded for TLC. The chromatogram was developed in rectangular glass chamber pre-equilibrated with the solution system for 60 min. The chromatogram was dried and a solution of the ninhydrin reagent was sprayed on the TLC sheet. Chromatogram was dried for 5 min at 110^0C. The RF of the tested sample spot was calculated and identified by comparing with RF of standard L-tryptophan [16].

Identification of the selected isolate
Morphological characterization:
Colony morphology of the isolate producing L- tryptophan was recorded after growing the strain on Davis and Mingioli minimal medium at 10^0C for 1 to 3 days. Overall shape, size, pigmentation, opacity, elevation, margin; whether smooth, rough, mucoid or glossy were recorded as per Smibert & Krieg [17].

Cell morphology was observed under high power magnification of a Leica microscope (Model D-100) after Gram staining. Cellular morphology was also determined by Scanning Electron Micrograph (SEM) following Poli *et al*. 2006 [18].

Biochemical Characterization
Physico-biochemical characters i.e. Indole production, Methyl Red test, Voges Proskauer test, Citrate utilization, Acid-gas production from glucose, Catalase activity, Oxidase activity were checked following standard procedure [17].

The optimum growth temperature was determined by incubating the strain in different temperature (0, 4, 8, 10 and 15 ^{0}C).

Utilization of different carbohydrates
Carbohydrate utilization pattern was tested using kit available from HiMedia, India. 15 µl of actively growing culture suspension of the isolate was inoculated in each of the carbohydrate wells and incubated at 10^0C for observing any visible colour changes.

Study of antibiotic sensitivity
The antibiotic sensitivity test was done by disc diffusion method [19]. Zone of inhibition was recorded after growing the culture at 10^0C for 48-96 hr. The antibiotics used were tetracycline (30), nancomycin (30), erythromycin (15), genatamycin (5), nalidixic acid (30), kanamycin (30), chloramphenicol (15) and cotrimoxazole (10).

DNA isolation, 16S rRNA gene amplification, sequencing and phylogenetic analysis
Genomic DNA was isolated following the method of Marmur [20]. Amplification of 16S rRNA gene was carried out using primers 27f (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). Amplification, purification of the amplicon and sequencing of the 16S rRNA gene were done as described by Pandey *et al*. [21]. A near complete (1391 nucleotide long continuous stretch) sequence of 16S rRNA gene of the strain was used for phylogenetic analysis with different psychrophiles belonging to phylum Proteobacteria, Firmicutes, Actinobacteria and Bacteriodes. The phylogenetic tree was constructed using MEGA 4 software. DNA G+C contents of the strain 023K was determined following protocol of Shivaji *et al*. [22]

Quantitative estimation of L-tryptophan
Quantitative estimation of L-tryptophan was carried out spectrophotometrically following Hassan [23]. Residual sugar in the culture filtrate was estimated by the dinitrosalicylic acid method [24]. Protein content was determined by the Bradford method, with bovine serum albumin (BSA) as a standard [25].

Selection of a suitable medium for L-tryptophan production
In order to select suitable medium for production of L-tryptophan by the selected psychrophilic strain 023K, five different mineral salt mediums were tested including Davis and Mingioli's medium. Twenty milliliter of each medium was taken in separate 100 ml Erlenmeyer flasks, sterilized and inoculated with washed bacterial cells of Strain 023K (100 µl contained $7.5x10^4$ cells.) from 3 days old broth culture. Growth, protein content [25], unspent glucose [24] and L-tryptophan production [23] were measured simultaneously.

Time course L-tryptophan production
In order to study the progress of fermentation, the selected bacterial strain was inoculated in the selected medium (20 ml) in 100 ml Erlenmeyer flask. Growth, tryptophan production, protein content, pH level and residual sugar were analyzed at each 24 hours interval.

Statistical analysis: All data presented are average of three replicates. Standard error and P-values were calculated using STATISTICA software.

RESULTS

Isolation of strain for L-tryptophan production
Soil samples from Nalbandpora, Kashmir, India, were serially diluted and plated on glucose minimal medium agar plates and incubated at 10^oC for isolating psychrophilic bacterial strains. After incubation for three days 22 different psychrophilic bacterial colonies were obtained from 10^{-2} and10^{-3} dilution of those samples. Out of 22, 3 strains viz. B05K, B08K and 023K from soil sample were selected on the basis of extracellular accumulation of L-tryptophan in the growth medium. Strain 023K showed maximum L-tryptophan production and thus selected for further studies. Qualitative estimation of L-tryptophan was done by TLC method (Fig 1).

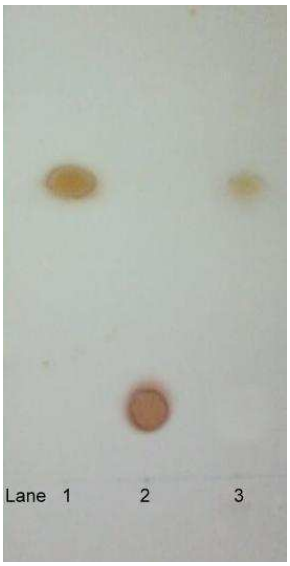

Fig 1. TLC pattern of amino acid produced in culture medium by strain 023K after 72 hrs in Davis and Mingioli medium. Lane 1: Standard L-tryptophan , Lane 2: Standard serine, Lane 3: Test sample

Morphological and Physio-biochemical Characteristics of strain 023K
Morphological and physio-biochemical characterization were done from 48 hr old culture. Light microscope (Fig 2a) and SEM (Fig 2b) observation showed that the isolated bacterial strain is a rod shaped (1-1.5 × 0.2-0.4) gram negative one. The purity of the isolate was assessed by colony morphology and microscopy after 48 hours growth on minimal agar plate. Morphological characteristic of the strain 023K shows the colonies were small, yellowish, with smooth edges. Biochemical characteristics of the strain are given in the Table 1. Optimum growth temperature was found to be 10°C (Fig 3).

Fig 2- Photomicrograph of the isolated bacterial strain 023K.(a) Gram stained cells under high power magnification of Light microscope (100x). (b) SEM photograph at 8000X

Table 1. Biochemical characters

Character	Response
Indole production	-
Methyl Red test	+
Voges Proskauer test	-
Citrate utilization	-
Acid production from glucose	+
Oxidase	+
Amylase activity	+
Gas production from glucose	-
Catalase activity	+
Gelatin hydrolysis	+
Lipase	-

(+) = positive for the test; (-) = negative for the test.

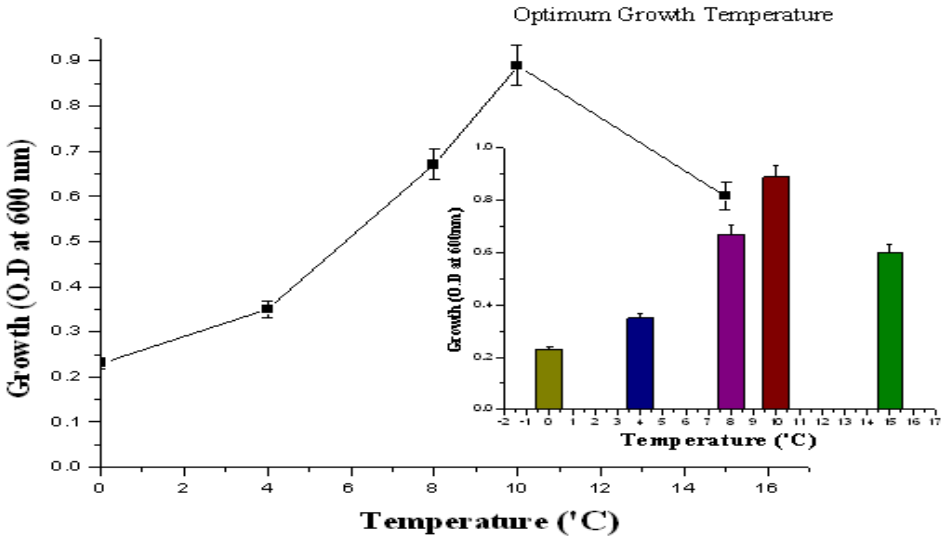

Fig 3. Effect of temperature on growth of isolate 023K

Carbohydrate utilization test

Ability of utilization of various types of carbohydrates by the selected strain 023K was studied as described in the materials and methods section. The results reveals that although some sugar-alcohol substrates (like trehalose, salicin, esculin, inositol, mannitol, dulcitol etc.) can not be utilized, the test strain can utilize most of the common mono and disaccharide substrates such as lactose, dextrose, maltose, mannose, ribose, galactose, sucrose, arabinose, sorbose, cellobiose and rhamnose.

Antibiotic sensitivity profile

Antibiotic sensitivity of strain 023K was tested against 8 conventional antibiotics. The inhibition zone against all the antibiotics suggested that the isolate 023K was susceptible to all the tested antibiotics.

Identification on the basis of 16S rRNA sequence analysis:

Analysis carried out by using 16S rRNA gene sequence of the strain 023K and various online tools available at RDP site suggested that the strain belongs to the genus *Pseudomonas*, and named as *Pseudomonas* sp. 023K. The Neighbour-joining phylogenetic tree (Fig.4) clearly indicates that it forms a clade within the cluster represented by *Pseudomonas chlororaphis*. The strain showed closest sequence similarity with *Pseudomonas chlororaphis* ATCC 13985 (100%) followed by *Pseudomonas putida* NBRC 12653 (99%). The 16S ribosomal RNA gene of *Pseudomonas sp*. 023K, was submitted to GenBank and the following accession numbers was assigned for isolate 023K is JX878339. DNA G+C content of strain 023K was found to be 66.23 mol%.

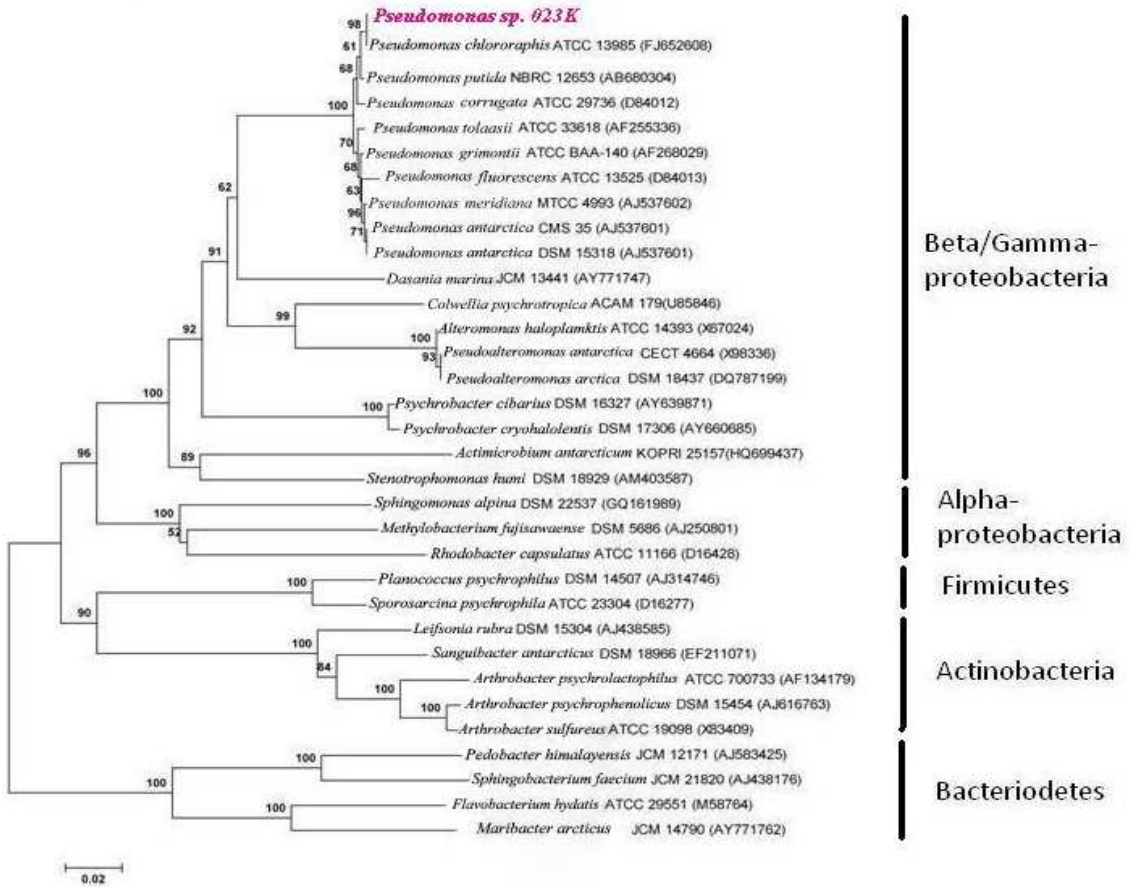

Fig 4. Phenogram showing the phylogenetic relationship between isolated bacterial strain 023K and other psychrophiles belonging to Proteobacteria, Firmicutes, Actinobacteria and Bacteriodes

Selection of a suitable medium for L-tryptophan production

Five different types of mineral salt medium, as described in material and methods section were tested for their suitability as production medium. Growth, protein content, unspent glucose and L-tryptophan production were measured in the five test production mediums simultaneously (Fig.5). Though all the four parameters were studied, Tokoro [26] medium was selected as best production medium considering growth and L-tryptophan production. Though DM* medium [15] and Alfoldi's medium [27] shows good growth and glucose consumption by the strain, the tryptophan production was not satisfactory .The medium of Tanaka [28] and Robinson (modified) [29] were not suitable for tryptophan production as well.

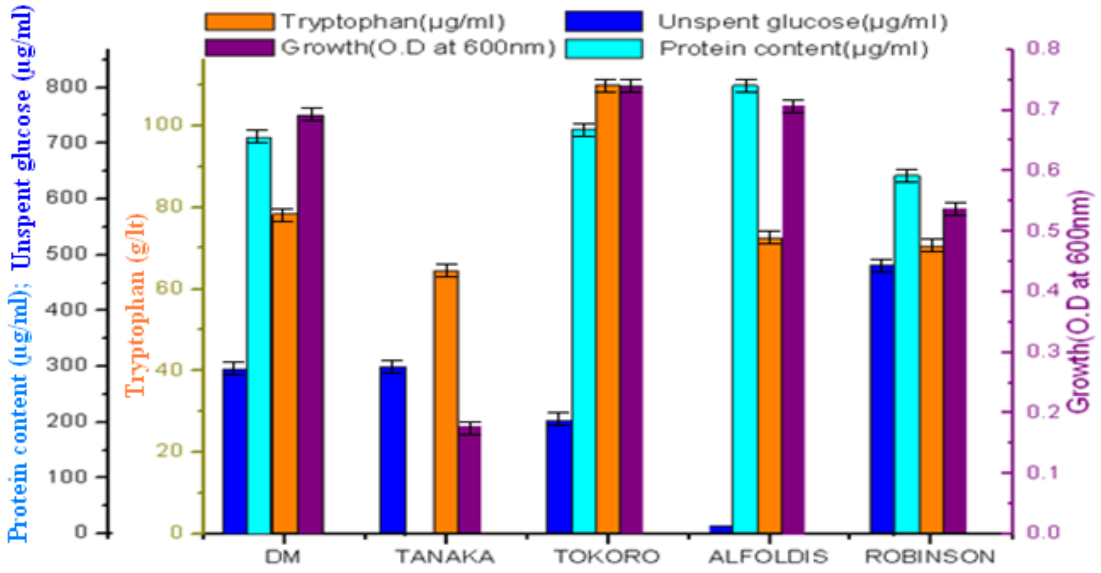

Fig 5. Suitability of different mineral salt media for growth and Tryptophan production (DM*, Tokoro, Tanaka, Alfoldi's and Robinson modified). * DM indicates Davis and Mingioli medium

Time course L-tryptophan production in Tokoro medium

The time course of growth and the production of L-tryptophan were studied using Tokoro medium (Fig.6). From the figure it clear that the isolated bacterial strain 023K entered stationary phase in about 96 hr to 120 hr in the medium. Extracellular accumulation of L-tryptophan was first detected in the medium after 24 hrs of growth and increases till 168 hr. During late phase of growth curve, growth ceases gradually with continuous consumption of glucose and tryptophan was excreted. A maximum L-tryptophan accumulation in the medium was observed after 72 to 120 hrs of growth. As the fermentation proceeds, pH of the medium becomes acidic from initial value of 7.2 to 6.8. The result shows that the time of incubation, cell growth and glucose has significant ($p<0.05000$) effects on L-tryptophan production.

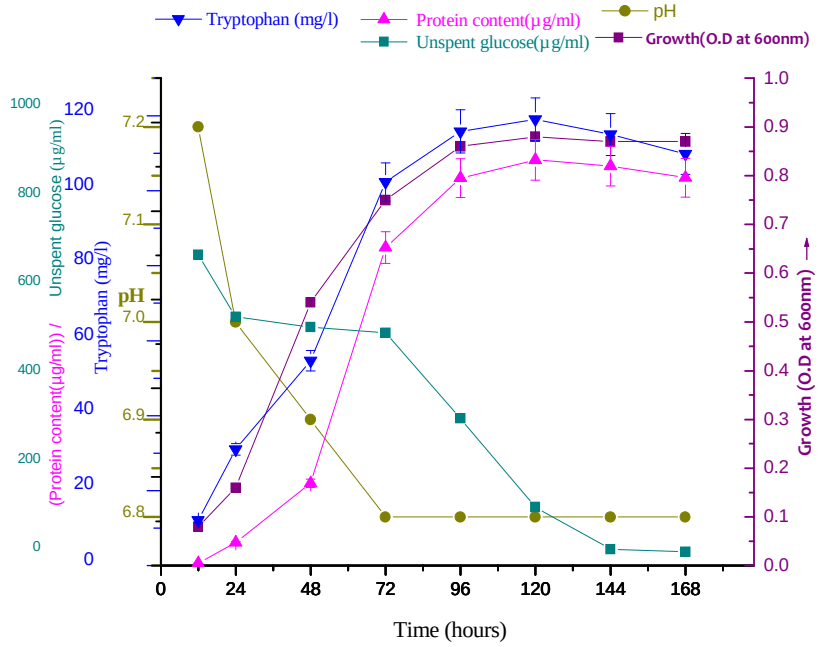

Fig 6. Time bound course of growth and L-tryptophan production by strain 023K in Tokoro medium

Correlations (Spreadsheet1)

Marked correlations are significant at p < 0.05000

N=8 (Case wise deletion of missing data)

	Incubation	Growth	Tryptophan	Glucose
Incubation	1.000000	0.860355	0.878372	-0.970234
Growth	0.860355	1.000000	0.994922	-0.761851
Tryptophan	0.878372	0.994922	1.000000	-0.794620
Glucose	-0.970234	-0.761851	-0.794620	1.000000

DISCUSSION

At present, the global demands for amino acids are achieved by microbial fermentation. Annual demand of L-tryptophan for use as feed additives and in pharmaceutical products is huge. Demand of L-tryptophan as food additive is increasing significantly in countries like India, where staple food of majority of population is cereals which naturally contain very low or no tryptophan [11]. Though L-tryptophan production by many mesophilic and thermophillic bacteria is reported, there are no such reports of L-tryptophan production by any psychrophilic bacterial strain. In this study we have isolated a psychrophilic bacterial strain capable of producing L-tryptophan extracellularly. Phylogenetic analysis based on 16s rDNA sequence homology reveals that this strain 023K belongs to the genus *Pseudomonas*, and named as *Pseudomonas* sp. 023K. The genus *Pseudomonas* was originally created by Migula [30]. The psychrophilic strain *Pseudomonas* sp. 023K shows optimum growth at a temperature of 10°C. This may be useful for producing L-tryptophan in temperate countries in a much economical way. Strong sensitivity to conventional antibiotics and non-haemolytic nature(data not shown) indicate that this strain can be used safely for industrial purpose. Optimization result shows that strain 023K significantly increases the production of L tryptophan in Tokoro medium among the five tested medium. Rapid increase in protein concentration and decrease in sugar content of medium, during the growth demonstrate active metabolism of Strain 023K. It was observed that during the progress of fermentation the pH of the medium gradually become acidic and after 72 hrs it became 6.8 from pH 7.2. As any significant pH change is not observed during the course of growth in the production medium, necessity of pH control, which is a costly affair, is not required. This makes the strain more useful industrially. The uniqueness and the importance of this study lies in L-tryptophan production extracellularly in cheap medium and at low optimum temperature. L-tryptophan used till date has an optimum production temperature at 30^0C average, making its production and use costly in temperate countries (85% of total biosphere), where the average ambient temperature is below 5^0C throughout the year [31]. From our result it is evident that the isolated strain 023K has tremendous potentiality to be exploited industrially in those regions.

CONCLUSION

In conclusion, the newly isolated psychrophilic wild type strain *Pseudomonas* sp. 023K shows potential L-tryptophan producing ability in cheap mineral salt medium. The production may be further increased by careful optimization of other parameters and as well as by inducing suitable mutation.

Acknowledgement
We thankfully acknowledge the Honbl' Vice Chancellor, Burdwan University for providing necessary infrastructural facilities.

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