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Isolation and characterization of amino acid producing bacteria from cow dung

Moni Krishno Mohanta*, Mst. Sabia Aktar Mohua, M. Saiful Islam, Md. Fazlul Haque and Ananda Kumar Saha

Genetic Engineering Laboratory, Department of Zoology, University of Rajshahi, Rajshahi 6205, Bangladesh
Email ID: mkmohanta_zool@yahoo.com

Abstract

Isolation and characterization of three distinct amino acid producing bacteria from the cow dung (CD) suspensions under aerobic condition have been studied. Based on their morphological and biochemical characteristics, the isolates of white colour bacteria (WCB), red colour bacteria (RCB) and yellow colour bacteria (YCB) were identified to be Gram-positive, rod-shaped and non-motile microbes belonging to *E. coli*, *Bacillus* sp.1 and *Bacillus* sp.2, respectively. The growth conditions of the bacterials isolated were then studied in the laboratory and the corresponding pH and temperature ranges were determined. Results on the antibiogram profiles of the bacterial isolates showed that 80-90% of the bacteria were sensitive against the antibiotics tested during the study. Amino acid producing capability of the bacteria was finally assessed in molasses-based fermentation media, and the amino acids isolated and identified from the three types of bacteria using paper chromatography were cysteine, serine and methionine. The relevance of the findings in relation to the commercial production of amino acids of medicinal, agricultural and nutritional significance has been discussed.

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Corresponding address: Genetic Engineering Laboratory, Department of Zoology, University of Rajshahi, Rajshahi 6205, Bangladesh
Email ID: mkmohanta_zool@yahoo.com

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Introduction

Amino acids are the most important source of energy which is crucial for the metabolic activities and thus play important role in various physiological processes in all living organisms. In addition, these are the building blocks of proteins, constitute a major part of the body, involved in building cells and repairing tissues, and form antibodies to combat foreign bodies like bacteria and viruses (Shakoori *et al*., 2012). Amino acids have a wide variety of applications in many different ways; they are used as animal feed additives (Campbell, 2001), some are flavour enhancers while others are used for therapeutic and psychotherapeutic purposes (Akashui *et al*., 1979; Kinoshita, 1987; Hermann, 2003).

Cow dung (CD) is bovine excreta that contain a mixture of dung and urine, generally in the ratio of 3:1. It contains crude fibre, crude protein, cellulose and various types of minerals such as N, K, S, and traces of P, Fe, Co, Mg, P, Cl and Mn (Nene, 1999). CD micro-flora usually contains abundant number of bacilli, lactobacilli and cocci and some identified and unidentified fungi and yeasts (Muhammad and Amusa, 2003) that can play an important role in producing enzymes, amino acids and other biomolecules.

Teo and Teoh (2011) identified five distinct morphologically and physiologically bacterial isolates from CD where all the isolates produced protease, lipase and esterase lipase. In a couple of recent studies, researchers have produced clear evidence that CD is an excellent source of biogas production owing to its methanogenic bacteria (Gopinath *et al*., 2014) and CD is capable of releasing major amino acids (Chomini *et al*., 2015).

In several recent studies, CD has been shown to be a cheap and available bio-resource that harbours a diverse group of microorganisms which may be beneficial to humans due to their ability to produce a range of metabolites. Thus cellulase producing bacteria (Bai *et al*., 2012; Hong-li *et al*., 2015), methanogenic bacteria (Pradhan and Gireesh Babu, 2012), indole acetic acid and ammonia producing bacteria (Radha and Rao, 2014) have been isolated and identified from CD. Moreover, bacterial isolates from CD having enzymatic activities (Sharma and Singh, 2015; Vijayaraghavan *et al*., 2016), and CD microbes capable of bio-fuel production and combating environmental pollutants (Gupta *et al*., 2016) have been reported, which imply that CD could be harnessed for medicinal, agricultural and industrial usages. Keeping the aforesaid findings in mind, the present investigation was aimed at isolating, characterizing and identifying amino acid producing bacteria from CD of exotic Jersey (Australian) cows at Rajshahi, Bangladesh.

Material and Methods

Collection of samples

The samples of the CD were collected from lactating Jersey cows of a dairy farm located at Gosh Para, Kazla, Rajshahi. Soon after collecting the CD sample in sterile polythene bags, they were transported to the Genetic Engineering Laboratory, Department of Zoology, Rajshahi University, for further processing and analyses.

Enrichment of culture for amino acid producing bacteria

The CD samples were suspended in individual 250 ml Erlenmeyer flaks each containing 100 ml of Luria-Bertani (LB) medium. Control flasks without an inoculum were also maintained for comparisons. The primary enrichment was incubated for two days at 30°C with shaking at 120 rpm on an orbital shaker. The cultures that were found turbid after a period from 0 to 2 days were used as inocula in subsequent experiments.

Microscopic examinations and identification of bacterial cells

For the identification of the bacteria, morphological characters, microscopic observations, growth characteristics, biochemical tests and antibiotic sensitivity tests were performed. The microorganisms were identified using *Bergey's Manual of Determinative Bacteriology* (Holt *et al*., 2005).

Effects of temperature and pH on bacterial growth

Since both pH and temperature have been found to influence bacterial growth (Pradhan and Gireesh Babu, 2012; Radha and Rao, 2014), the present nutrient broth culture media (Hi-media, India) were adjusted to pH 5.0, 6.0, 7.0 and 8.0, and the incubation temperatures were varied at 25°C, 30°C, 37°C and 40°C to study growth characteristics of the bacterial isolates. Bacterial cell density of the nutrient broth was determined by measuring optical density at 660 nm with a photoelectric colorimeter (AE-11M, Erma Inc., Tokyo) following the procedures described by Mohanta *et al.* (2012).

Screening of bacterial isolates for amino acid production

Media formulation: For the production of amino acids, molasses-based fermentation media (MF media) were tested for the isolation of amino acid fermenting bacteria obtained from the CD samples. The ingredients for the 100 ml media were: KH_2PO_4 (0.05g), K_2HPO_4 (0.05g), $MgSO_4.7H_2O$ (0.025g), $(NH_4)_2SO_4$ (2.00g) and $CaCO_3$ (2.00g). Agricultural industrial waste (10g.100 ml^{-1} cane molasses) was used in the media as carbon source. The pH of the media was kept within a range of 7.0-7.2. Sterilization was done through autoclaving the media at 121°C at 15 lbs pressure for 15 min.

Fermentation: A loopful of bacterial culture from a desired plate was inoculated into 250 ml Erlenmeyer flask containing 50 ml of the fermentation medium. The flask was incubated at 30°C on a rotary shaker. The experiments for amino acid producing potential of these bacterial strains were carried out in a rotary shaker at 120 rpm for a maximum period of 96 hrs. During this period, 3 ml sample was monitored every 24 hrs. The harvest pH of each flask was also noted during the 96-hr incubation period.

Analysis and identification of the fermented amino acids

About 3 ml sample of fermented broth was centrifuged at 15000 rpm for 10 min in order to collect the cell-free broth. Qualitative analyses of amino acids produced by bacteria in this fermented and cell-free broth were performed as detailed below.

Qualitative analysis using paper chromatography

Lederer and Laderer (1957) method was followed for paper chromatography, where 0.03M standard solutions of six amino acids *viz*., methionine, serine, leucine, proline, cysteine and glycine were prepared. Solvent made of *n*-butanol: acetic acid: water (at 4: 1: 1) was added in a chromatographic rectangular glass jar. Standard amino acids and samples, 50 µl each, were loaded on to the Whatmann I chromatographic papers (Desaga Nr. 2045). The chromatographic tank papers were irrigated vertically in the solvent system for few hrs until the solvent traveled the distance on filter paper up to a certain point. Then the papers were air dried and sprayed with 0.1% ninhydrin solution (0.1g.100 ml^{-1} ethanol), and later dried at 60-80°C for 10 min to get purple spots of the amino acids. The results were confirmed by comparing the retention factors (R_f values) of the samples with that of the standard amino acids. The R_f values were calculated by the following formula:

$$Rf = \frac{Distance\ traveled\ by\ amino\ acid}{Distance\ traveled\ by\ solvent}$$

The distance was measured from the point where the amino acid was loaded to the point where solvent came to a halt.

Results

Isolation and identification of the bacteria

Bacteria were isolated by plating onto an agar solidified nutrient medium.

The plates were incubated at 30°C for a day and bacterial colonies were found to grow on the medium. Results of microscopic analysis of bacterial cells and their colony morphological characteristics are presented in Tables 1 (a) and 1 (b) whereas the biochemical tests of the isolates are presented in Table 2. Preliminary characterization of the isolates according to their morphological and biochemical tests indicated that the bacterial strains belonged to *E. coli*, *Bacillus* sp. 1 and *Bacillus* sp. 2.

Antibiogram profile of the bacterial isolates

The antibiogram profile analyses of the three bacterial isolates revealed that 80-90% bacteria were sensitive whereas the remaining 10-20% bacteria were resistant against 10 antibiotics tested during the study (Table 3).

Effects of temperature and pH on bacterial growth

The growth of the bacterial isolates depended on pH and temperature. WCB and YCB showed optimum growth at pH 7 while the maximum growth of RCB was observed at pH 6 and the extreme pH (5.0 and 8.0) restricted for the growth of the bacteria (Figs.1, 2 and 3, respectively).

The optimum growth conditions of WCB and RCB, on the hand, were determined at 30°C while YCB showed maximum growth at 25°C (Figs. 4, 5 and 6, respectively).

It is apparent from the results that both the temperature and pH were important factors for the bacterial growth and so they will affect enzymatic reactions necessary for the production of amino acids from the CD suspensions.

Identification of amino acids

The maximum production of amino acids was obtained by the MF media used in the present experiment. The amino acids produced by the isolates in the media were identified by using paper chromatography followed by calculating their specific R_f values. Results presented in Table 4 and Fig. 7 demonstrated that the bacterial strains WCB, RCB and YCB produced the amino acids cysteine, serine and methionine, respectively.

Bacterial strains	Gram characteristic	Shape	Motility
WCB	+ ve	Rod	Non motile
RCB	+ ve	Rod	Non motile
YCB	+ ve	Rod	Non motile

Table 1(a). Microscopic observations of the isolated bacterial strains

WCB= white colour bacteria; RCB= red colour bacteria; YCB= yellow colour bacteria

Bacterial strains	Colony morphology					
	Colour	Shape	Surface	Elevation	Edges	Opacity
WCB	White	Irregular	Smooth	Umbonate	Undulate	Opaque
RCB	Red	Circular	Smooth	Convex	Entire	Clear
YCB	Yellow	Circular	Smooth	Convex	Entire	Clear

Table 1(b). Colony morphology of the isolated bacterial strains

WCB= white colour bacteria; RCB= red colour bacteria; YCB= yellow colour bacteria

Tests performed	Isolates		
	WCB	RCB	YCB
Triple sugar iron (TSI) test	+	+	+
Citrate utilization test	-	-	-
Oxidase test	+	+	-
Catalase test	-	+	+
Sulfide indole motility (SIM) test	-	-	-
Methyl red test	-	+	-
MacConkey agar test	-	-	-
Carbohydrate utilization tests			
Fructose	-	-	-
Galactose	-	-	-
Lactose	-	-	-
Arabinose	-	-	-
Maltose	-	-	-
Xylose	-	-	-

Table 2. Biochemical test results for the isolated bacterial strains

WCB= white colour bacteria; RCB= red colour bacteria; YCB= yellow colour bacteria;

+ = microbial growth; - = no growth

Antibiotic discs	WCB Disc distance (mm)	RCB Disc distance (mm)	Disc distance (mm)
Ampicillin (10µg)	41 (S)	39 (S)	8 (R)
Azithromycin (15µg)	21 (S)	29 (S)	17 (S)
Cephradine (30µg)	21 (S)	26 (S)	32 (S)
Ceftazidime (30µg)	6 (R)	6 (R)	6 (R)
Doxycycline (30µg)	16 (S)	20 (S)	22 (S)
Erythromycine (15µg)	32 (S)	32 (S)	16 (S)
Gentamicin (10µg)	40 (S)	41 (S)	34 (S)
Kanamycin (30µg)	10 (R)	13 (I)	30 (S)
Neomycin (30µg)	20 (S)	22 (S)	31 (S)
Tetracycline (30µg)	16 (S)	17 (S)	23 (S)

Table3. Antibiotic sensitivity tests

WCB= white colour bacteria (20% R); RCB= red colour bacteria (10% R); YCB= yellow colour bacteria (20% R); (5-10mm) = Resistant to antibiotics (R); (11-15mm) = Intermediate resistance (I); (>15mm) = Sensitive to antibiotics (S).

Bacterial strains	R_f values	Amino acids
WCB	0.64	Cysteine
RCB	0.30	Serine
YCB	0.77	Methionine

Table 4. Amino acids produced by the bacterial strains

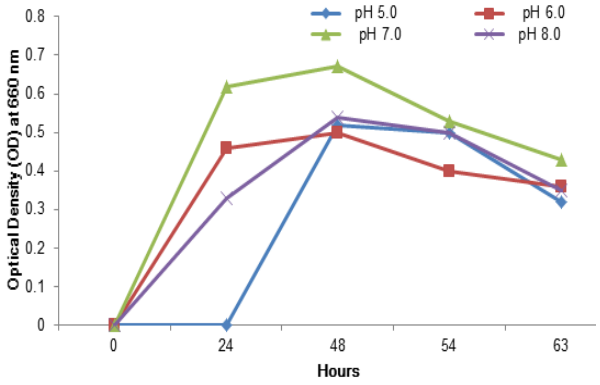

Figure 1: Effect of pH on the white colour bacterial isolates (WCB)

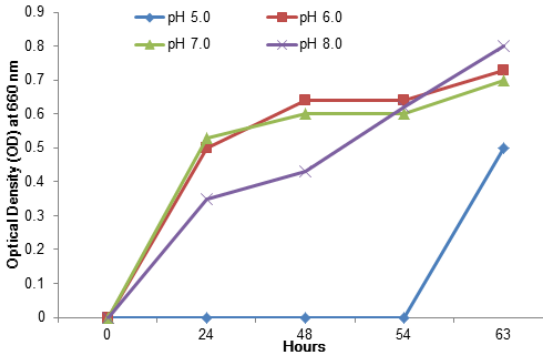

Figure 2: Effect of pH on the red colour bacterial isolates (RCB)

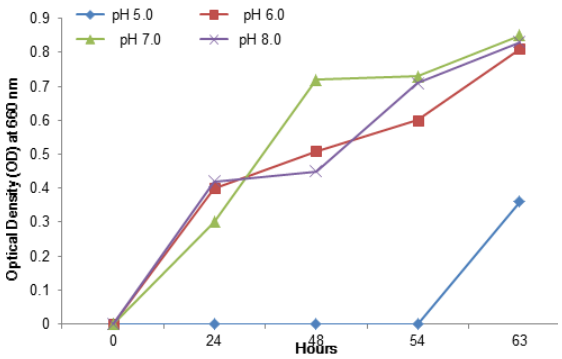

Figure 3: Effect of pH on the yellow colour bacterial isolates (YCB)

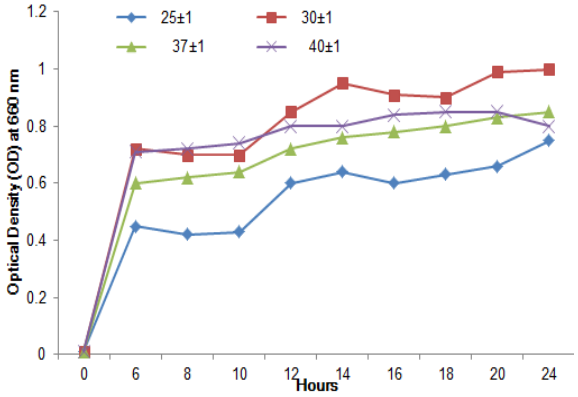

Figure 4: Effect of temperature on the white colour bacterial isolates (WCB)

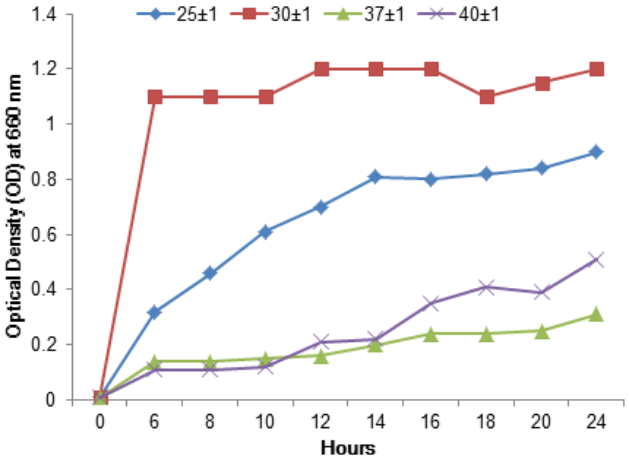

Figure 5: Effect of temperature on the red colour bacterial isolates (RCB)

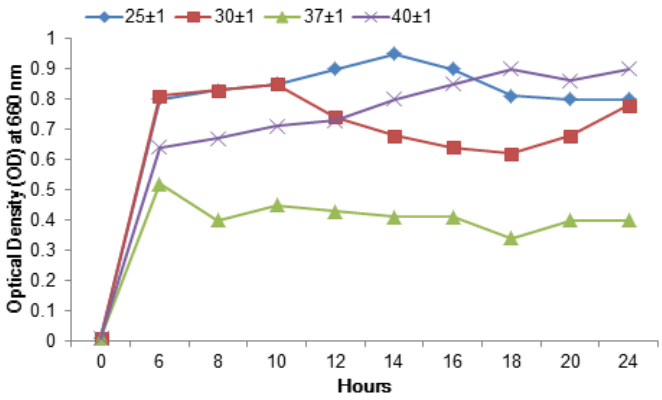

Figure 6: Effect of temperature on the yellow colour bacterial isolates (YCB)

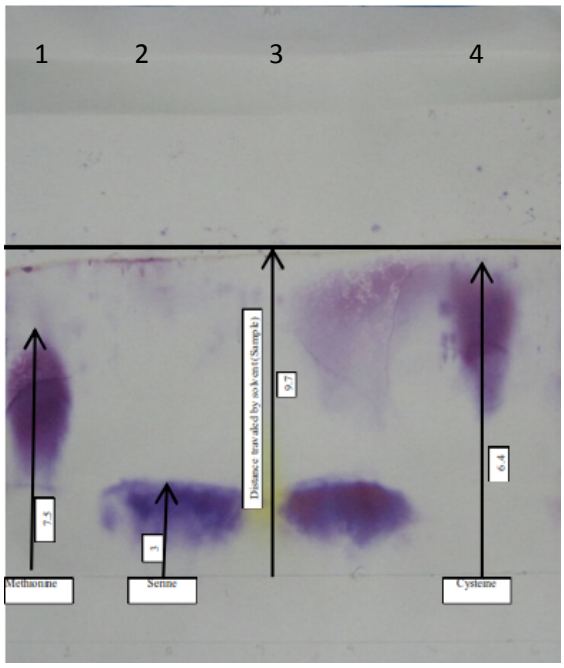

Figure 7: Paper chromatography of amino acids isolated from three bacterial strains *viz.*, YCB, RCB and WCB (lanes 1, 2 and 4). The paper was sprayed with ninhydrine solution and dried at 60-80 °C for 10 min to get purple spots of the amino acids. Lane 3 was the control.

Discussion

In the present study three distinct amino acid producing bacteria were isolated from the CD suspensions at 30°C under aerobic condition. Based on their morphological and biochemical characteristics, the isolates WCB, RCB and YCB were identified to be Gram-positive, rod-shaped and non-motile bacteria belonging to *E. coli*, *Bacillus* sp.1 and *Bacillus* sp.2, respectively. The optimum growth conditions of the bacterials isolated were then studied in the laboratory and the corresponding pH and temperature ranges were determined.

Results on the antibiogram profiles of the bacterial isolates showed that maximum of the bacteria were sensitive against the antibiotics tested during the study. The amino acid producing capability of the bacteria was finally assessed in molasses-based fermentation media, and the amino acids isolated and identified from the three types of bacteria using paper chromatography were cysteine, serine and methionine.

Antibiotic sensitivity tests of the present study slightly differ from those of Teo and Teoh (2011) who used Kirby-Bauer assay to evaluate susceptibilities of five isolates to 17 different types of antibiotics, where each individual isolate was resistant to at least 35% of the antibiotics tested. In a recent study, eight antimicrobial drugs were used to treat the susceptibility patterns of isolated bacteria from CD; among four isolates two showed resistance to penicillin and rest of the strains were sensitive to all the antibiotics (Sharma and Singh, 2015). This is in good agreement with those of the present results.

Cysteine production by bacterial isolates in different fermentation media has been documented by Ali *et al*. (2011). Moreover, Gopinath *et al*. (2014) isolated different bacterial species from CD and reported that it contained high amount of methanogenic bacteria that increased its efficiency of biogas production. Recently, Chomini *et al*. (2015) demonstrated that four major amino acids *viz*., threonine, proline, glycine and alanine were released from digested CD. However, the present findings, corroborate to Shakoori *et al*. (2012) where *B. cereus* was found to produce cysteine and glutamic acid, *E. coli* produced valine, and cysteine and methionine were produced by *B. anthracis*.

The right conditions for the growth of bacteria are present in a cow's stomach. These bacteria are able to produce all the essential amino acids, even though its feed may not contain a full set of amino acids (Majda, 2014). For examples, Fungsin *et al*. (2008) isolated 240 strains of lactic acid bacteria *Lactobacillus salivarius*, and Khan *et al*. (2011) diagnosed endospore producing Gram-positive cocci from samples of CD. Teo and Teoh (2011) isolated five morphologically and physiologically distinct isolates, two Gram-negative and three Gram-positive, from CD that produced enzymes such as protease, lipase and esterase lipase. Cellulase producing *Bacillus subtilis* (Bai *et al*., 2012) and methanogenic *Bacillus* sp. and *Proteus* sp. (Pradhan and Gireesh Babu, 2012) were identified using 16S rDNA sequencing and BLAST search. In addition, indole acetic acid and ammonia producing *Bacillus* spp. and *Lysinibacillus xylanilyticus* (Radha and Rao, 2014), cellulase producing *Stenotrophomonas* sp. and *Bacillus cereus* (Hong-li *et al*., 2015), and strains of Gram-positive cocci and gram-negative bacilli (Sharma and Singh, 2015) were identified from CD. Recently, Gupta *et al*. (2016) and Vijayaraghavan *et al*. (2016) utilized CD microbes for bio-fuel production and management of environmental pollutants, and fibrinolytic enzyme production, respectively. So far the bacterial genera isolated from the CD are concerned; the present findings are in line with most of the work cited above, particularly Bai *et al*. (2012), Pradhan and Gireesh Babu (2012); Radha and Roa (2014); Hong-li *et al*. (2015) and Vijayaraghavan *et al*. (2016).

Amino acids are of great nutritional importance in food. In general, the world at present is confronted with the serious problems of food and nutrition deficiencies. Increased demand of proteins has led the researchers to search for unconventionally sources of proteins and amino acids (Majda, 2014; Vijayaraghavan *et al*., 2016). One of such sources is the microbes, particularly bacteria present in the CD which are capable of producing amino acids in profuse quantities (Fungsin *et al*., 2008; Radha and Rao, 2014; Chomini *et al*., 2015). The results of the present study are quite encouraging in that the fermented bacterial suspensions of the CD could be utilized on commercial scale in the country, with special reference to their agricultural, medicinal and nutritional significance.

Conclusion

In summary, we conclude that the microbial fermentation process can be used for the production of amino acids at very economical rates by using locally isolated strains from natural sources. The natural source including CD (cowdung) which is rich in micro-flora can provide bacterial strains for production of amino acids by fermentation on commercial scale to meet the local demand in the country.

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