International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 10, Issue 4, 2018, pp.-1155-1159.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

Research Article

ISOLATION AND CHARACTERIZATION OF POTENTIAL CELLULYTIC BACTERIA FROM WHEAT RHIZOSPHERE AMENDED WITH RICE STRAW

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Received: April 20, 2018; Revised: April 24, 2018; Accepted: April 25, 2018; Published: April 30, 2018

Abstract- In this study, seventeen bacterial cultures were isolated and screened for cellulose degradation activity. The maximum halozone was observed with SKPB3 (2.8 mm). These isolates were then studied morphologically and most of them showed sticky consistency. Biochemical characterization showed most of them are oxidase and catalase positive and negative for H_2S test, indole test and MR-VP test and few were positive for citrate utilization, gelatin solubilization and starch hydrolysis. The functional characterization of isolates showed highest cellulolytic activity (1.44 µg/ml), IAA production (34.85µg/ml), ammonia excretion (2.048µg/ml) and phosphate solubility (28.56 µg/ml) was exhibited by isolate SKPB3. The results revealed that the wheat field incorporated with rice straw contained bacteria which had ability to degrade the cellulose or they have ability to utilize the cellulose.

Keywords- Biochemical characterization, cellulolytic bacteria, cellulolytic activity, morphological characterization and rice straw

Citation: Bhagat Poonam and Gosal S.K. (2018) Isolation and Characterization of Potential Cellulytic Bacteria from Wheat Rhizosphere Amended with Rice Straw. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 10, Issue 4, pp.-1155-1159. DOI: http://dx.doi.org/10.9735/0975-5276.10.4.1155-1159

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Introduction

Crop residue is the richest source of organic polymers as the plant cell wall is made up of cellulose. The rice straw could be used as organic manure to improve the organic carbon of the soil for enhanced crop production. The cellulose degradation has a significant role in recycling of nutrients in the soil environment and relies mainly on the soil micro-organisms who have ability to degrade cellulose [1]. The degradation of cellulose is happened by the action of enzyme called cellulase. Cellulases are mainly hydrolytic in nature and divided into three major types: β-1,4-endoglucanases (EC 3.2.1.4), β-1,4- exoglucanases and β-glucosidases (EC 3.2.1.21) [2]. Cellulase enzyme has much importance because of its diverse application in various fields [3,4].

The exploration of micro-organisms that that have high ability to degrade cellulose is much in demand. Cellulolytic enzymes from microbes have many potential biotechnologic and industrial applications [5]. Most of the research regarding cellulases has been concentrated on fungi but due to high growth rate, there is increasing interest in cellulose production by bacteria [6]. The cellulases from bacteria are more effective catalysts. The present research is related with the isolation and identification of some soil bacteria having cellulose degrading activity depending morphological, physiological, biochemical and functional characterization. The present research was conducted with the objective of isolation of potential cellulose degrading bacteria that could be used further in improving agroecosystem.

Material and Methods

The rhizospheric (0-15cm) soil samples were collected from the wheat field having rice straw incorporation since 2005. The samples were serially diluted and spread plated on carboxymethyl cellulose (CMC) agar [7] to isolate cellulose degrading bacterial colonies. These isolated colonies were screened by flooding with 0.1% congo red for 20 minutes and then washed with 1M NaCl [8]. The halozone formation confirmed the cellulolytic activity of isolates. The isolated colonies were picked up, purified then maintained on nutrient agar at 4ºC [9]. The preliminary characterization of isolates was done by morphological, physiological, biochemical and functional characterization. The biochemical characterization includes tests such as oxidase, catalase, H_2S production, indole, methyl red, Simmon's citrate and Voges Proskaeur according to Bergey's Manual of Systematic Bacteriology [10]. The functional characterization includes indole acetic acid production [11], ammonia excretion [12], phosphate solubilization [13]. The other functional test includes zinc solubilization, siderophore production [14].

Results and Discussion

Cellulolytic activity of potential isolates

Seventeen morphologically distinct isolates were screened on the basis of halozone formation as indication of cellulose degrading activity. The superlative cellulolytic activity among isolates was examined by measuring the clear zone diameter to colony diameter ratio when incubated at 37ºC aerobically. The highest ratio was considered to produce the maximum cellulolytic activity. In the present investigation the zone to colony diameter ratio ranges from 1.6 to 2.8 mm and the maximum ratio of zone to colony diameter [Fig-1] was observed with isolate SKPB3 (2.8) followed by zone ratio 1.6 by SKPB2 and SKPB5. Similar to present results, clear zones were observed by Apun, *et al*. [15]. Similarly, Teather and Wood, [8] proved that congo red dye have particular interaction with the cellulosic substrates. The work of Howard, *et al*., [16], Ariffin, *et al*., [17], Huang, *et al*., [18] found more cellulolytic activity with the isolates having high zone ratio. The similar findings for isolated cellulose degrading bacteria were described by Behera et al [19] who observed the ratio of zone size to colony size ranged between 1.18 to 2.5 cm and Hatami, *et al*., [20] who reported zone the ratio between 0.4 to 2.1, but high ratio was observed by Lu, *et al*., [21] who ranged the ratio between 2.5 to 6.4 cm and Gupta, *et al*., [22] who observed maximum ratio ranged between 9 to 9.8 cm.

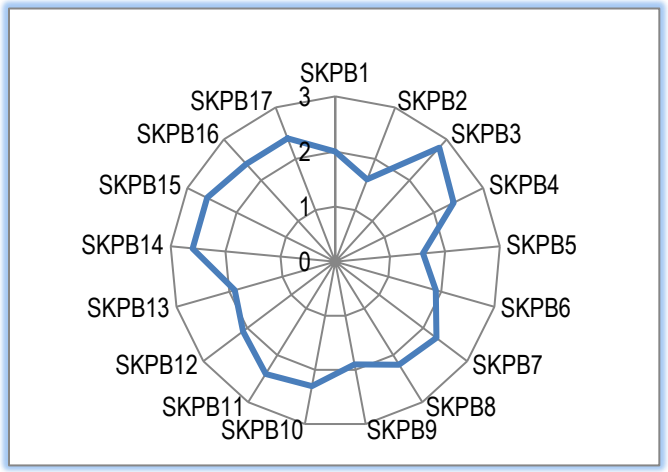

Fig-1 Ratio of zone size to colony size

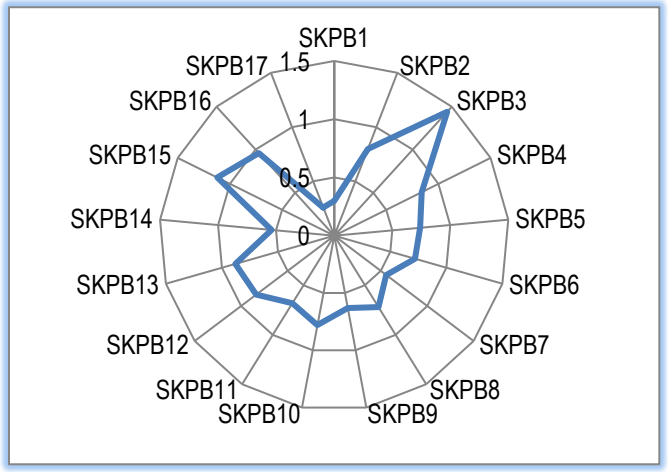

Fig-2 Cellulolytic activity by isolates

The seventeen isolates were quantitatively determined for cellulolytic activity [Fig-2]. The maximum cellulolytic activity revealed by isolate SKPB3 (1.44 µg/ml) followed by SKPB16 (1.12 µg/ml), SKPB16 (0.96 µg/ml), SKPB13 (0.88 µg/ml). The least cellulolytic activity was observed with isolate SKPB17 (0.26 µg/ml). The findings were in accordance to Pourramezan, *et al*., [23] who also recorded cellulolytic activity of various isolates and rangend from 0.24 to 1.47 U/ml. The work of Bahera, *et al*., [19] also demonstrated the cellulolytic activity of potential bacteria and ranged from 2.471 to 18.253 U/ml/min.

Morphological and biochemical characterization of potential isolates

The morphological characterization of isolated colonies was observed to be variable. The most of the isolated colonies were spherical, undulated, convex and were observed to be gummy and sticky in consistency. Also, the most of the isolated colonies were showed average size *i.e*., between 0.2 to 0.5 cm. The isolated bacteria were observed microscopically and found that most of them are motile in nature and showed rod shape morphology. The gram staining of these isolated cultures showed that most of the isolates were gram negative and few of isolates were found to be gram positive [Table-1]. The similar observations for cellulose degrading isolates *i.e*., circular, convex undulated, sticky and motile colonies were observed by Behera et al [19].

The seventeen isolates were identified based on standard biochemical characterization. The biochemical characterization was done by analyzing basic tests that include catalase, oxidase, methyl red, V-P, indole test, urease production, utilization of citrate, starch and gelatin hydrolysis, H_2S test. All the isolates were found to be catalase positive except SKPB17, approximately fifty percent isolates showed oxidase test positive and fifty percent showed oxidase test negative. All the isolates exhibited negative test for H_2S production, only SKPB3 gave indole test positive rest isolates showed negative test, most of the isolates were able to utilize citrate, except SKPB3 all isolates gave MR-VP test negative, few of the isolates showed positive test for urease production, gelatin solubilization and starch hydrolysis [Table-2]. The work of Behera, *et al*., [19] also showed biochemical characterization of cellulose degrading bacteria as preliminary way for the identification of cellulose degrading bacteria. Similar observations regarding biochemical characterization of cellulose degrading bacteria were reported by Huang, *et al*., [18], Pourramezan, *et al*., [23], Ahmad, *et al*., [24].

Functional characterization of potential isolates

In the present investigation, the IAA was studied in the presence of tryptophan and in the absence of tryptophan and found to be ranged from 20.46 to 34.85 (µg/ml) and 6.28 to 18.56 (µg/ml) respectively. The maximum amount of IAA production with tryptophan was observed in the isolate SKPB3 (34.85 µg/ml). The maximum amount of IAA production without tryptophan was also found in isolate SKPB3 (18.56 µg/ml). The graphical representation showed that in the presence of tryptophan [Fig-3A] 18% of isolates exhibited high IAA production (31-35 µg/ml), 53% showed low IAA production (20-24 µg/ml) and 29% of isolates exhibited medium amount of IAA (25-30 µg/ml). The graphs were also showed for IAA production in the absence of tryptophan [Fig-3B], 29% of the isolates observed to produce high amount of IAA (15-20 µg/ml), 18% found to produce low amount of tryptophan (0-10 µg/ml) and rest 53% of isolates produce medium amount of IAA (10-15 µg/ml). The present results were in accordance with Gazala [25] who reported production of 10-35 µg/ml IAA in a medium supplemented with tryptophan. Nadeem, *et al*., [26] also found the production of 15-19 µg/ml by various soil micro-organisms. The findings of Bhatia, *et al*., [27] exhibited that various soil bacteria able to produce IAA in presence of tryptophan. The various researchers Dias, *et al*., [28], Mishra, *et al*., [29], Bhromsiri and Bhromsiri, [30] reported that there was more IAA production in the rhizospheric soil micro-organisms. The ammonia excretion from the isolated cellulose degrading bacteria was determined and it was observed that the present isolates were ranged from 0.528 to 2.048 µg/ml. The maximum amount of ammonia excretion was exhibited by isolate SKPB3 *i.e*., 2.048 µg/ml. The graphical representation [Fig-3C] showed that 18% of isolates excreting high amount (1.9-2.7 µg/ml) of ammonia, 23% of isolates excrete lower amount of ammonia (0-0.9 µg/ml) and rest 59% isolates excrete medium amount of ammonia (1.0-1.8 µg/ml). Hartono, *et al*., [31] noticed positive reaction for ammonia excretion by change in colour of the medium to reddish brown showing ammonia excretion by isolates. The reports of Bhatia, *et al*., [27] also reported ammonia excretion from isolates ranged from 3.03-13.06 µg/ml. Similar results were obtained by Verma, *et al*., [32] who reported the production of ammonia by rhizospheric soil micro-organisms.

The phosphate solubilization quantity by cellulose degrading bacterial isolates in the present investigation ranged from 18.24-28.56 µg/ml. The isolate SKPB3 exhibited maximum phosphate solubility 28.56 µg/ml. The graphical representation [Fig-3D] of the isolates for phosphate solubilization exhibited that 24% isolates revealed high phosphate solubilization ability (25-28 µg/ml), 41% exhibited low phosphate solubilization ability and rest 35% exhibited medium phosphate solubilization ability (21-24 µg/ml). The present results were supported by Hariprasad and Niranjana, [33], Thanuja and Ambika, [34] who also measured phosphate solubilizing ability by different soil microbes. Mishra, *et al*., [29] observed higher number of phosphate solubilizer microbes in the rhizopheric zone as compared to non rhizospheric zone, the results were in agreement of the present observations which studied rhizospheric soil micro-organisms.

The other functional characters that determined in this study exhibited that only four of the isolates exhibited siderophore production and rest of the isolates were negative for siderophore test. The graphical representation showed that [Table-2] 24% isolates exhibited positive results for siderophore production while 76% showed negative results. The ammonia production by the cellulose degradation bacterial isolates was studied and observed that 82% of the isolates exhibited positive results for this test. The isolates were studied for zinc solubilization and it was observed that 71% of the isolates gave negative test and 29% gave positive result for zinc oxide while 53% gave positive and 47% gave negative results for zinc phosphate [Fig-3G & 3H].

Table-1 *Morphological and physiological characterization of isolated cellulose degrading bacterial colonies*

Isolates	Colony size	Colony shape	Colour	Margin	Elevation	Density	Pigmentation	Motility	Gram stain	Spore formation	Growth
SKPB1	Average	Oval	off white	Undulated	Convex	Viscuous	NIL	+	-	+	Aerobic
SKPB2	Average	Spherical	pale yellow	smooth	Convex	Mucoid	NIL	-	-	-	Aerobic
SKPB3	Average	Spherical	off white	Undulated	Convex	Slimy	NIL	+	+	+	Aerobic
SKPB4	Average	Spherical	off white	smooth	Convex	Slimy	NIL	-	-	-	Aerobic
SKPB5	Average	Spherical	red	smooth	Convex	Slimy	Red	+	-	+	Aerobic
SKPB6	Average	Spherical	off white	Undulated	Flat	-	NIL	+	-	-	Aerobic
SKPB7	Average	Spherical	pale yellow	Undulated	Flat	Slimy	NIL	+	+	-	Aerobic
SKPB8	Small	Spherical	pale yellow	Undulated	Convex	-	Yellow	+	-	-	Aerobic
SKPB9	Average	Spherical	pale yellow	entire	Convex	-	NIL	-	-	-	Aerobic
SKPB10	Average	Spherical	pale yellow	Undulated	Flat	Slimy	NIL	+		-	Aerobic
SKPB11	Small	Spherical	off white	Undulated	Flat	Slimy	NIL	+	-	-	Aerobic
SKPB12	Average	Spherical	pale yellow	entire	Convex	Glistening	NIL	+	-	-	Aerobic
SKPB13	Average	Spherical	pale yellow	Undulated	Flat	Slimy	NIL	-	-	-	Aerobic
SKPB14	Small	Spherical	off white	Undulated	Convex	-	NIL	+	-	-	Aerobic
SKPB15	Average	Spherical	off white	Undulated	Convex	Slimy	NIL	+	-	-	Aerobic
SKPB16	Average	Spherical	pale yellow	Undulated	Convex	Slimy	NIL	+	-	-	Aerobic
SKPB17	Small	Spherical	pale yellow	Undulated	Convex	Viscous	NIL	+	-	-	Aerobic

Table-2 *Biochemical and functional characterization of isolated cellulose degrading bacterial colonies*

	Catalase	Oxidase	H_2S production	Indole test	Citrate utilization	MR test	VP test	Urease test	Gelain solubilization	Starch hydrolysis	Siderophore production	Zinc solubilization		Ammonia production
												Zinc Oxide	Zinc phosphate	
SKPB1	+	+	-	-	-	-	-	-	-	-	-	-	-	+
SKPB2	+	-	-	-	-	-	-	-	-	-	-	-	-	+
SKPB3	+	+	-	+	+	+	+	-	+	+	+	+	+	+
SKPB4	+	-	-	-	+	-	-	-	-	-	-	+	+	+
SKPB5	+	-	-	-	+	-	-	-	+	-	-	+	+	+
SKPB6	+	-	-	-	-	-	-	-	+	+	-	-	+	+
SKPB7	+	-	-	-	-	-	-	-	-	+	-	-	+	+
SKPB8	+	+	-	-	+	-	-	-	+	-	+	-	-	-
SKPB9	+	+	-	-	+	-	-	+	-	+	-	-	+	+
SKPB10	+	+	-	-	+	-	-	+	-	+	-	-	-	+
SKPB11	+	-	-	-	-	-	-	-	+	+	-	+	-	+
SKPB12	+	+	-	-	+	+	-	-	-	-	-	+	-	+
SKPB13	+	-	-	-	+	-	-	-	-	-	-	-	-	+
SKPB14	+	+	-	-	+	-	-	-	-	-	+	-	-	-
SKPB15	+	+	-	-	+	-	-	+	-	+	+	-	+	+
SKPB16	+	+	-	-	+	-	-	+	-	+	-	-	+	-
SKPB17	-	-	-	-	+	+	-	-	-	-	-	-	+	+

The results were similar to the studies of Zamin, *et al*., [35] who also observed functional characters of the rhizospheric soil mcro-organisms. Gupta and Gopal, [36] also exhibited similar results from the soil microbes for functional characterization. The work of Baura, *et al*., [37] also showed various functional characterization tests for micro-organisms found in the rhispheric zone of plants.

Conclusion

The present research concluded that the seventeen potential cellulose degrading bacteria which are able to utilize the agrowastes as energy substrate were isolated using CMC agar plates. The halozone formation with congo red showed the cellulolytic activity of the potential cellulose degrading bacteria. The colony to zone diameter ratio showed highest by the isolate SKPB3 indicating highest cellulolytc activity among all isolates. The catalase and oxidase tests were positive for most of the isolates while H_2S test, indole test and MR-VP test showed negative results for maximum number of isolates. The citrate utilization, gelatin solubilization and starch hydrolysis was observed to be positive for few isolates. The fine results for functional characteristics were given by isolate SKPB3.

Application of research

The agricultural waste is used as sunbstrae for the proliferation of soil microorganisms and these soil micro-organisms are useful for the higher production of the crop.

Research Category: Integrated farming, cellulose degrading bacteria

Abbreviations:

CMC: Carboxymethyl Cellulose

Acknowledgement / Funding: Author thankful to Punjab Agricultural University, Ludhiana, Punjab, 141003, India

***Research Guide or Chairperson of research: Professor Dr S K Gosal**
University: Punjab Agricultural University, Ludhiana, Punjab, 141003, India
Research project name or number: Nil

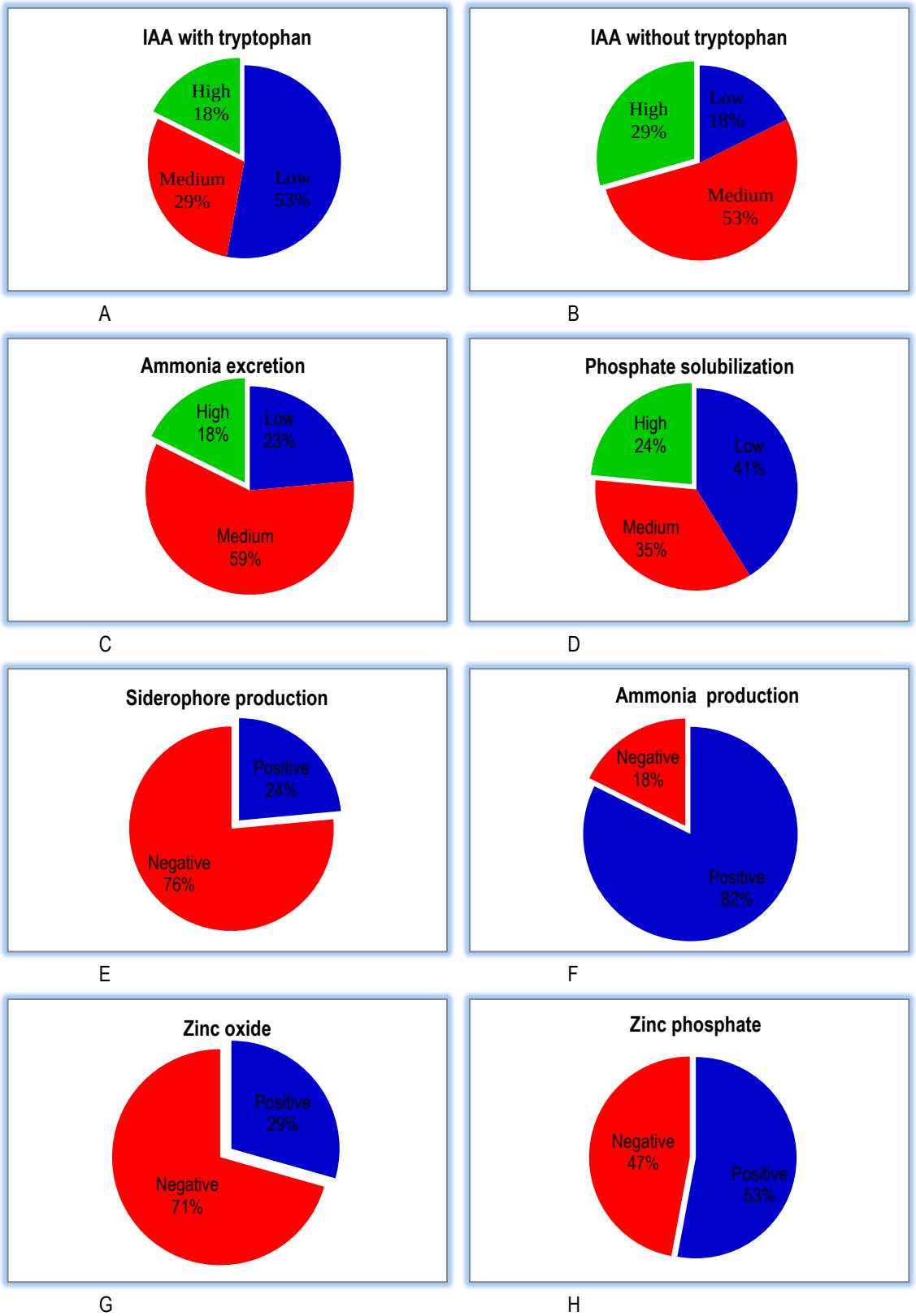

Fig-3 Functional characterization of isolates (A) IAA production with tryptophan (B) IAA production without tryptophan (C) Ammonia exretion (D) Phosphtae solubilization (E) Siderophore production (F) Ammonia production (G) Zinc solubilization on ZnO containing medium (H) Zinc solubilization on ZnP containing medium

Author Contributions: All author equally contributed

Author statement: All authors read, reviewed, agree and approved the final manuscript

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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