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Purification, characterization, and antiproliferative activity of L-methioninase from a new isolate of *Bacillus haynesii* JUB2

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ABSTRACT

L-methioninase (MGL) has received much attention in the recent years, since it shows antiproliferative activity and methionine restriction has been known to arrest tumor growth. Methionine dependence is the only known general metabolic defect in cancers. Normal cells have the ability to grow on homocysteine, instead of methionine, due to their active methionine synthase. Thus, depleting cellular/plasma methionine levels using MGL seems to be a promising therapeutic approach to treat cancer. Therefore, the objective of the present study is isolation of soil microbes using the selective M9 media for MGL producers and screening them for MGL activity. The results showed a novel bacterial strain, identified as *Bacillus haynesii* JUB2, which exhibited highest specific activity of 9.22 U/mg of protein. This enzyme was purified by acetone precipitation and diethylaminoethyl column methods. Upon column purification, fraction 6 yielded the highest enzyme activity and the specific activity increased enormously to 102.15 U/mg of protein. The cytotoxicity 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide assay performed on breast (MCF-7) and liver cancer cell lines (HepG2) showed highest cytotoxicity with a percentage viability of 63.2 and 68.6, respectively, at 250 µg/ml concentration. It can be concluded that purified MGL from the new source of *B. haynesii* JUB2 holds promise toward anticancer studies.

INTRODUCTION

L-methioninase (EC 4.4.1.11; MGL), also termed L-methionine-γ-lyase, is a pyridoxal phosphate (PLP)-dependent hydrolytic enzyme which is known to catalyze the γ-elimination of L-methionine to generate α-ketobutyrate, methanethiol (MTL), and ammonia, as well as the α, β-replacement and β-elimination of S-substituted L-cysteines (Tanaka *et al*., 1983). MGL is a cytosolic enzyme inducibly formed by the addition of L-methionine to the culture medium (Lockwood and Coombs, 1991). It has a molecular weight between 149 and 173 kDa and consists of four subunits with identical molecular weights of about 41–45 kDa each; the purified enzyme has been reported to have a molecular mass of 47 KDa (Nakayama *et al*., 1984).

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In a healthy environment, mammalian cells possess the ability to proliferate on homocysteine, instead of methionine, due to their innate ability to produce methionine synthase (Mecham *et al*., 1983). In contrast, tumor cells lack efficient methionine synthase and thus rely on external methionine supplementation from the diet (Hoffman, 1984). Cancer cells also have an elevated requirement for methionine (MET) in comparison to normal cells. This phenomenon is termed MET dependence (Hoffman, 2015). Methionine restriction has been known to arrest tumor growth and to induce a selective S/G2-phase cell-cycle arrest of cancer cells both *in vitro* and *in vivo* (Guo *et al*., 1993; Hoffman and Jacobsen, 1980; Kokkinakis *et al*., 1997a; Kokkinakis *et al*., 1997b). Studies have also reported the disruption and abnormalities in methionine metabolism and transmethylation to be associated with the major disease in humans, like obesity, Parkinson's disease, heart disease, and cancer (Kreis and Hession, 1973).

Compounds and enzymes from natural sources have played vital roles in cancer drug discovery (Ashrini and Varalakshmi, 2016). Microbial MGL has received much attention in the recent years, since it shows antiproliferative activity toward

various types of malignant cells (Cellarier *et al*., 2003). MGL is also one of the few microbial enzymes which possesses high therapeutic value, since its reporting as a potent anticancer agent against various types of tumor cell lines in breast, lung, colon, kidney, and glioblastoma (Kokkinakis *et al*., 2001; Tan *et al*., 2010). Tan and team reported the effective inhibition of the Yoshida sarcoma and human lung tumor with minimal toxicity in *in vivo* nude mice models after intraperitoneally injecting them with MGL (Tan *et al*., 1996). Thus, through many studies reporting activity of MGL, it has received affordable attention and has been widely studied for its various therapeutic potential, such as in cancer therapy and as antimicrobial drug (Alshehri, 2020; El-Sayed, 2011; Maggi and Scotti, 2019; Suganya *et al*., 2017), as a therapeutic agent against various types of methionine-dependent tumors.

MGL is present in most organisms, such as bacteria, fungi, protozoa, and plants. However, the enzyme is absent in mammals (Bhupender *et al*., 2014). MGL is produced as intracellular enzymes in most of the bacterial species. Both gram-positive and gram-negative bacteria have been reported to produce MGL (Bhawana and Priyanka, 2018). Among prokaryotes, MGL is widely distributed in bacteria, especially in *Pseudomonas* spp., and is induced by the addition of L-methionine to the culture medium. From the studies reported so far, the bacteria appear to be the potent prokaryotes for enzyme production.

Current debate revolves around the low therapeutic efficiency and high levels of toxicity caused by a few bacterial MGL which increases the demand for new bacterial MGL producers, with lesser side effects and more efficiency (Kudou *et al*., 2007). Considering this as the main aim, we have screened a number of bacteria from various soil sources for their MGL activities. Among the different isolates, the bacteria belonging to *Bacillus* sp. was selected for the study. In the present work, we isolated, purified, and characterized MGL from *Bacillus haynesii* JUB2 and evaluated its *in vitro* anticancer activity.

MATERIALS AND METHODS

Sample collection and isolation of microbes

Various soil, gut (fish and lamb), and fecal (cow, lamb, and poultry) samples were collected from different locations in and around Karnataka, India. Serial dilution-plate method was used for the isolation of all the samples (Johnson *et al*., 1959). Nutrient agar medium was used for isolation of bacteria, containing (g/L): peptone, 5.0; beef extract, 3.0; NaCl, 5.0; and agar, 20.0. Czapek Dox Yeast agar medium was used for the isolation of fungi, containing (g/L): sucrose, 30.0; $NaNO_3$, 3.0; K_2HPO_4, 1.0; $MgSO_4$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; yeast extract, 5.0; and Agar, 20.0. The serially diluted plates were incubated at 37°C for 48 hours for bacterial isolates and at 28°C for 7 days for fungal isolates, and the developed pure culture colonies were further purified on their respective media.

Screening for methioninolytic bacteria and fungi

Qualitative rapid assay plate method was used to screen the bacterial and fungal isolates for their MGL productivities using modified M9 media containing (g/L): L-methionine, 5.0; glucose, 2.0; Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NaCl, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 0.01; and agar, 20.0. The final pH of the medium was adjusted to 7.0 and phenol red (0.007%) was added to the medium as an indicator just before autoclaving (Sundar and Nellaiah, 2013a). The M9 plates were incubated at 37°C for 48 hours for bacterial isolates and at 28°C for 7 days for fungal isolates. Pink-colored colonies or growth forms resulting from the production of ammonia by the action of MGL on L-methionine were identified as MGL-producing isolates and were selected for further assays (Sundar and Nellaiah, 2013b).

L-methioninase assay

MGL activity was assayed according to a previously reported protocol. MTL produced from substrate L-methionine reacted with 5,5 dithio-bis-2-nitrobenzoicacid (DTNB; Sigma-Aldrich) to produce thionitrobenzoic acid which was detected at 412 nm using a spectrophotometer. The quantitative assay mixture in a final volume of 1 ml consisted of 20 mM of L-methionine in 0.05 M potassium phosphate buffer of pH 7.0, 0.01 mM PLP (Sigma-Aldrich), 0.25 mM DTNB, and 0.3 ml of culture supernatant of the microbial isolate (i.e., the crude enzyme sample). Controls comprised heat denatured (95°C for 30 minutes) culture supernatant. The developing yellow color of the assay mixture was read at 412 nm after an incubation period of 1 hour at 30°C. A sodium methane thiolate standard graph was used to determine the amount of MTL released. One unit (U) of MGL was expressed as the amount of enzyme that releases 1 µmole of MTL per minute under optimal assay conditions (Selim *et al*., 2015a).

Protein estimation

The protein concentration in the culture supernatant was estimated by Lowry's method (Lowry *et al*., 1951) using bovine serum albumin (200 µg/ml) as the standard, which was spectrophotometrically read at 660 nm.

Identification of the isolate

The best methioninolytic isolate was subjected to morphological and molecular characterization for identification. Preliminary identification was carried out based on microscopic and cultural observations by gram staining. Molecular identification of the promising bacterial isolate was performed by sequencing the conserved 16s ribosomal RNA.

L-methioninase enzyme purification

Partial purification of the enzyme was carried out by the acetone precipitation method (Nejadi *et al*., 2014). Five volumes of pre-chilled absolute acetone were added to one volume of the sample and vortexed for 30 seconds. The precipitate was separated by centrifugation at 8,000 rpm for 20 minutes at 4°C. Supernatants were discarded and 5 volumes of pre-chilled 50% acetone were added to pellets and vortexed for 30 seconds. The supernatant was discarded and the pellet was air-dried.

Ion exchange chromatography

The dialysate comprising partially purified enzyme was mixed with 50 mM acetate buffer (pH 5.0). The mixture was centrifuged and the clear supernatant was used for purification in ion exchange chromatography. The diethylaminoethyl (DEAE) cellulose column was pre-equilibrated with 50 mM acetate buffer (pH 5.0). After equilibration, the MGL fractions were eluted with a linear gradient of sodium chloride (50 mM–1 M) in the same

buffer. The active fractions were collected, pooled, and assayed for their activity and enzyme concentrations (El-Sayed, 2011).

Effect of temperature on the enzyme activity

The optimum temperature of the purified enzyme was determined by incubating the reaction mixture for 1 hour at various temperatures (25°C, 37°C, 40°C and 50°C), and then assaying their enzyme productivities. As there are many reports that say that the bacterial enzymes of *Bacillus* sp. best work in the range of 25°C–50°C (Agrawal *et al*., 2012; Rao and Narasu 2017; Yossana *et al*., 2006), these temperatures were chosen.

Effect of pH on the enzyme activity

The optimum pH of the purified enzyme was determined by using sodium citrate buffer pH 3.0–5.0, phosphate buffer pH 6.0–8.0, and tris-HCl buffer pH 9.0–10.0. The optimum pH of the enzyme was assayed by preincubating the enzyme solution at different pH values ranging from pH 3.0 to 10.0 for 1 hour at 37°C and then determining the enzymatic activity.

Screening for anticancer activity

The effect caused by the crude enzyme, lyophilized acetone precipitated, and column purified fractions of the promising isolates was checked on human breast cancer cell line (MCF-7) and human liver cancer cell line (HepG2) by conducting 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) cell viability assay. MTT assay was performed on treated and control cells. The cells were seeded in 96-well plates and incubated for 24 hours at 37°C in a 5% CO2 incubator for cell adhesion. 1, 10, 100, and 250 µg/ml concentrations of the crude and 1, 10, 100, 250, and 500 µg/ml concentrations of the lyophilized fractions respectively, were added in triplicates and incubated for 48 hours. After the incubation period, 100 µl of MTT solution was added to each well and the plates were incubated in the dark for 3 hours at 37°C, after which 100 µl of dimethyl sulfoxide was added to each well. The absorbance was recorded at 540 nm using an enzyme-linked immunosorbent assay plate reader (Mosmann, 1983). The percentage of cell viability was determined using the following formula:

Cell Viability % = Absorbance at 540 nm of the test sample / Absorbance at 540 nm of the control × 100

RESULTS AND DISSCUSSION

Screening for MGL production by bacteria and fungi

Screening of various bacterial and fungal samples for their methioninolytic activity yielded five bacterial and eight fungal isolates, distinguished by their pink-colored colonies on selective M9 medium as a result of the generation of ammonia by the action of MGL on the substrate L-methionine. The screening profile (Table 1) shows the ability of different bacterial and fungal strains to produce MGL, their activity, protein content, and specific activity.

Among the various isolates grown on M9 medium, bacterial isolate designated as B2 demonstrated highest enzyme activity. Furthermore, data illustrated (Table 1) clearly showed that the incubation period did not affect the enzyme production as the highest enzyme production was obtained after 2 days of incubation in bacterial isolates, as compared to 5 days among the fungal isolates. Bacterial isolates demonstrated better enzymatic activity in comparison to the fungal isolates. As bacteria B2 produced the enzyme with highest activity of 7.38 µmoles/min/mL and specific activity of 9.22 U/mg of protein, it was chosen as the best isolate for further experiments.

Identification of the isolate B2

Both gram-positive and gram-negative bacteria are known to be prolific producers of MGL (Bhawana and Priyanka, 2018). Preliminary identification was carried out based on morphological and colony characterization. The colonies were yellowish orange on nutrient agar (Fig. 1A) and pink colored on M9 medium. Microscopic evaluation by gram staining of the bacteria B2 showed purple single rods (Fig. 1B). Based on preliminary identification and morphological observations, the pigmented bacteria B2 was identified as gram-positive bacteria and to be a member of the *Bacillus* species.

Table 1. Screening for MGL production by bacterial and fungal isolates.

Isolates	MGL activity (µmoles/minute/ml)	Protein content (µg/ml)	Specific activity (U/mg protein)
Bacteria			
B1	0.11	800	0.13
B2	7.38	800	9.22
B3	2.00	800	2.50
B4	0.47	800	0.58
B5	0.35	800	0.43
Fungi			
F1	0.35	4,100	0.08
F2	0.23	4,700	0.04
F3	0.35	3,150	0.10
F4	0.58	3,700	0.15
F5	0.35	2,800	0.12
F6	0.35	2,850	0.13
F7	0.58	3,650	0.15
F8	0.23	2,700	0.08

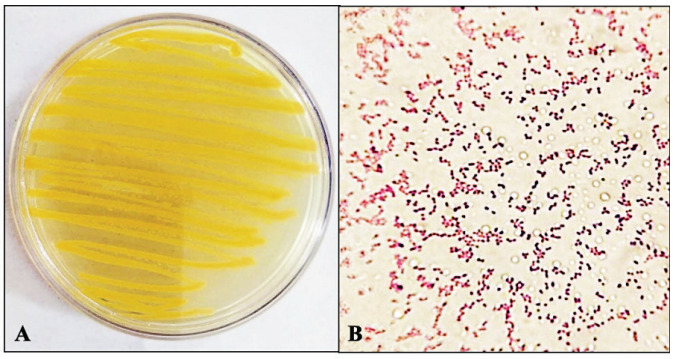

Figure 1. (**A**) The bacterial isolate (B2) pure culture on nutrient agar; (**B**) Microscopic view of gram-stained B2.

16s rRNA gene sequencing is a routinely used method for the identification of bacterial species (Mignard and Flandrois, 2006). PCR amplification of 16s rDNA fragment from bacterial isolate B2 generated a PCR amplified product of size ~1.5 kb (Fig. 2). Molecular identification further confirmed that the isolate belonged to the genus *Bacillus* and the 16S ribosomal RNA gene sequence analysis indicated that the bacterial isolate B2 was a novel strain of *B. haynesii*, exhibiting 99% similarity to *B. haynesii* strain NRRL B-41327. The phylogenetic tree showing the relatedness of the species is shown in Figure 3. This isolate was named as *B. haynesii* JUB2 (Sequence ID: NR_157609).

Purification of MGL

Acetone precipitation is an easy and reliable purification method employed in most laboratories (Crowell *et al*., 2013). MGL was precipitated from the broth culture of *B. haynesii* JUB2 using acetone, followed by ion exchange chromatography. The specific activity of MGL varied among the crude and the purified fractions.

Upon column purification, fraction 6 yielded the highest MGL activity of 19 µmoles/minutes/ml with a much higher specific activity of 102.15 U/mg in comparison to the crude enzyme (9.22 U/mg) and acetone purified fraction (0.018 U/mg). In a recent study (Abdelraof *et al*., 2019), it was reported that MGL was purified through Sephadex-G column resulting in a 3.15-fold purification. As compared to the report in the current study, our isolated bacterial MGL after DEAE column purification exhibited specific activity of 102 U/mg of protein that is a 11-fold higher than the crude enzyme activity (Table 2). In another study report, under optimized conditions *Bacillus subtilis* had exhibited a specific activity of 19.60 U/mg of protein (Bhawana and Priyanka, 2018), while the *Bacillus* species in the present study yielded a 5-fold higher MGL activity as compared to this activity.

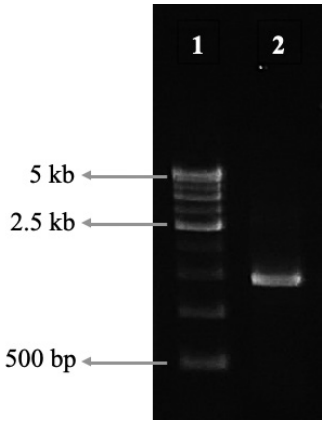

Figure 2. PCR amplification of 16s rDNA fragment from bacterial isolate B2. The size of PCR amplified product was ~1.5kb. Lanes 1: 500 bp DNA Ladder; Lane 2: B2 isolated DNA.

Biochemical properties of MGL

Optimum pH

The enzyme activity was assayed at different pH's ranging from pH 3.0 to 9.0. Among the different pH buffers checked, the activity of MGL was found to be highest in tris-HCl buffer of pH 8.0–9.0, yielding 1.42 µmoles/minutes/ml (Fig. 4). The control was set at pH 7.0 which yielded the least activity. In an earlier study, the productivity of MGL from *Aspergillus ustus* increased by increasing the alkalinity of the medium to reach maximum yield at pH 8.5 (Abu-Tahon and Isaac, 2016) comparable with the current study. The enzyme in our study did not work in a pH range between 3.0 and 5.0. However, in a very recent report (Mohkam *et al*., 2020), a novel bacterial isolate, *Alcaligenes* sp., had a pH optima of 6.0, i.e., in the acidic range.

Optimum temperature

Enzymes from bacterial sources usually work optimally at temperatures ranging between 25°C and 50°C or 55°C as

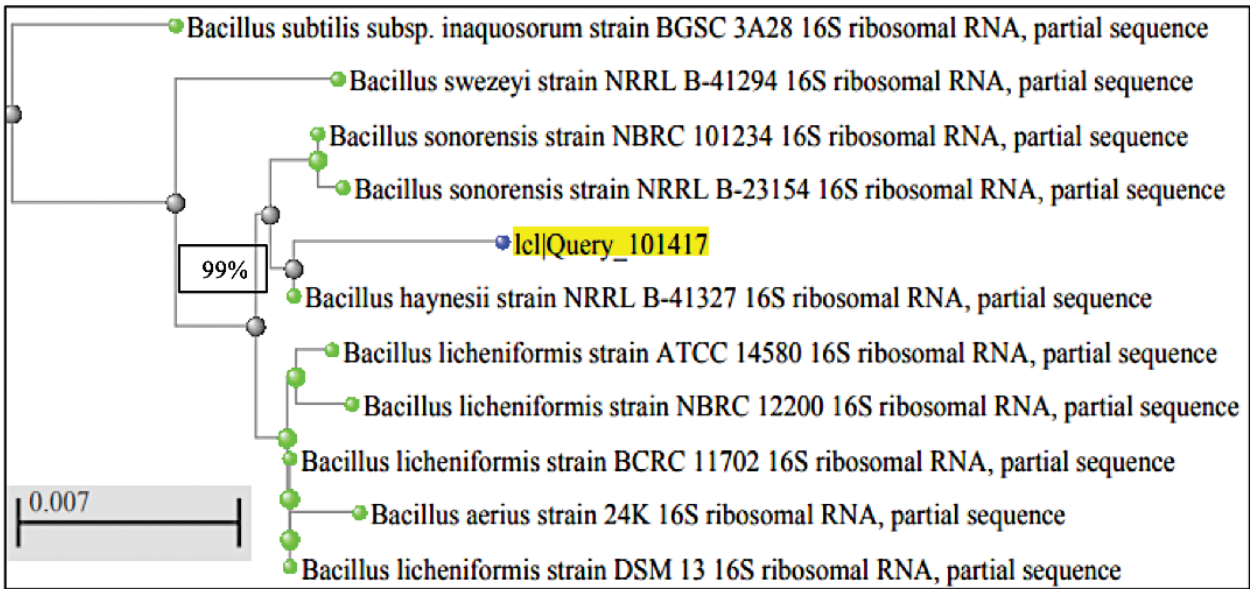

Figure 3. Phylogenetic analysis of the bacterial isolate B2.

per reported literature and our previous studies (Agrawal *et al*., 2012; Rao and Narasu 2017; Varalakshmi *et al*., 2012; Yossana *et al*., 2006). Hence, in the present work, when the enzyme mixture was incubated at different temperatures (25°C, 37°C, 40°C and 50°C), the highest MGL activity was observed at 37°C and second best was at room temperature (i.e., control) at 28°C ± 2°C. The activity decreased upon incubation at higher temperatures, remaining constant between 40°C and 50°C (Fig. 5). In accordance with the current study, MGL isolated from *Hafnia alvei* (Alshehri, 2020) and *Alcaligenes* sp. MT-B (Mohkam *et al*., 2020) under optimized conditions demonstrated the highest activity at 35C. In a previous report, the optimum temperature for the MGL enzyme activity of *Candida tropicalis* was 45°C (Selim *et al*., 2015b) and the optimum temperature

Table 2. Purification folds and activity of *B. haynesii* JUB2.

Enzyme	Enzymeactivity (µmoles/minute/ml)	Total protein (µg/ml)	Specific activity (U/mg protein)	Fold increase
Crude	7.38	800	9.22	1
Acetone purified	0.0085	0.450	0.018	–
Column purified	19	0.183	102.15	11.07

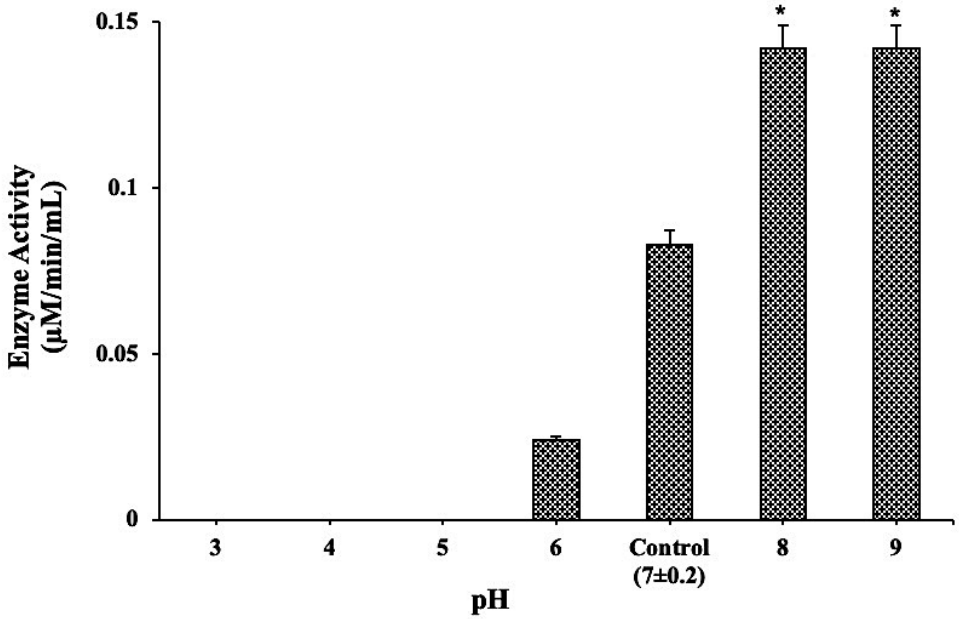

Figure 4. Effect of different pH's on MGL activity from *B. haynesii* JUB2 (*$p < 0.05$).

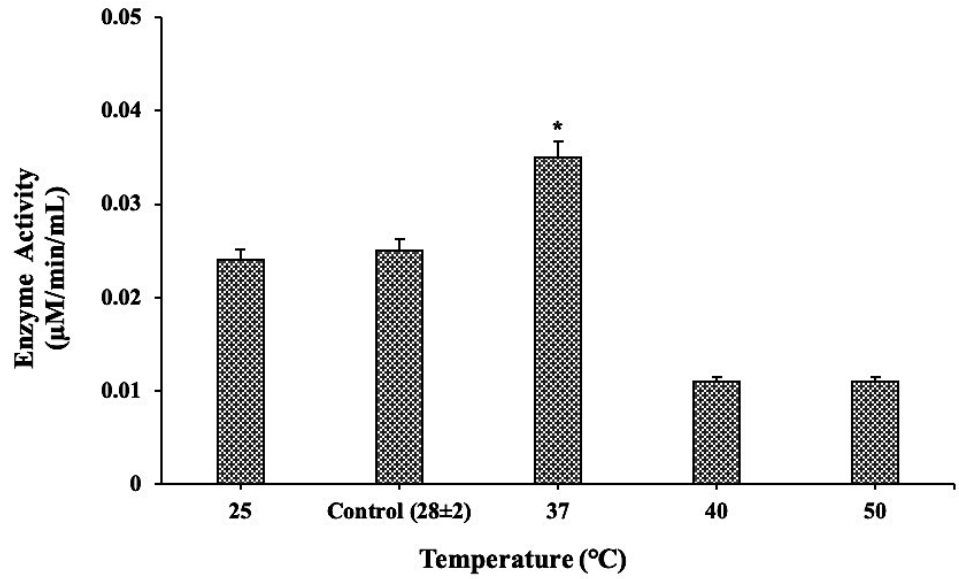

Figure 5. Effect of different temperatures on MGL activity from *B. haynesii* JUB2 (*$p < 0.05$).

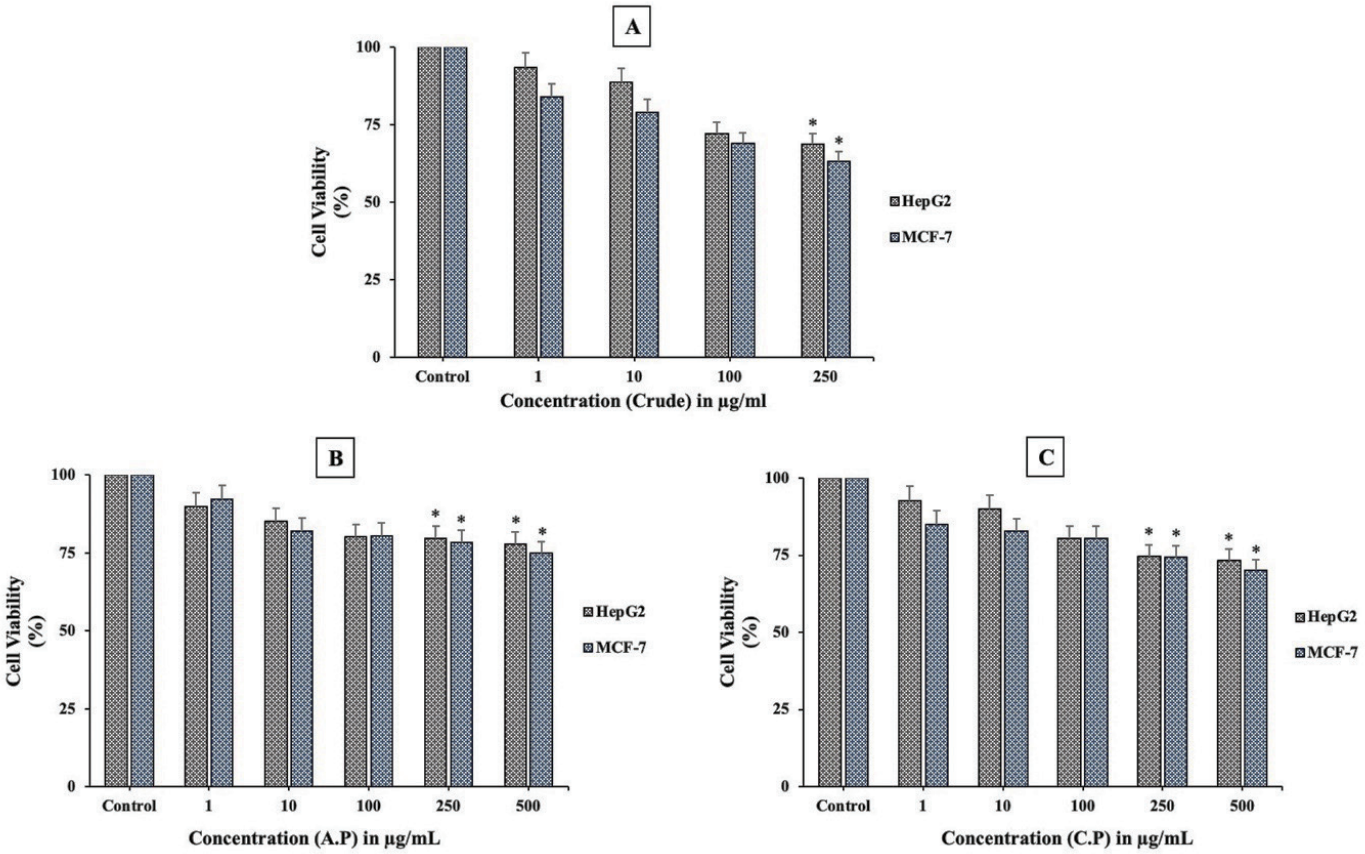

Figure 6. MTT assay of (**A**) crude extract; (**B**) acetone precipitate (A.P); and (**C**) column purified (C.P: Fraction 6) on HepG2 and MCF-7 cell viabilities when treated for 48 hours (*$p < 0.05$).

reported for MGL, purified from cheese lactic acid bacteria, was at 37°C (Hanniffy *et al*., 2009). All these studies are supportive of our observations about the temperature optima of MGL from *B. haynesii* in the current study.

Cytotoxicity studies

MTT assay

The efficiency of MGL against various cell lines was reported by many authors (Sundar and Nellaiah, 2013b; Tan *et al*., 2010). In the current study, cancer cells HepG2 and MCF-7 were treated with crude sample, acetone precipitated, and column purified (fraction 6) samples for 48 hours, as there are many studies that report and support the time period of either 24 or 48 hours to confirm the *in-vitro* anticancer potential of MGL (Huang *et al*., 2015; Selim *et al*., 2016). As per the results, the crude sample of MGL exhibited highest cytotoxicity with a percentage viability of 63.2 and 68.6 at 250 µg/ml concentration on MCF-7 and HepG2 cells, respectively (Fig. 6A). Column purified fraction 6 demonstrated a cell viability at 70% and 73.3% on MCF-7 and HepG2 cells, respectively, at a concentration of 500 µg/ml (Fig. 6C). The acetone precipitated sample yielded a cell viability of 74.9% and 77.8% on MCF-7 and HepG2 cells, respectively, at a concentration of 500 µg/ml (Fig. 6B). As compared to the purified enzyme samples, the crude sample was having higher cytotoxic effects on the treated cancer cell lines at lower treatment concentrations. We also observed that the effect of MGL on the viability of MCF-7 cell line was higher than that on HepG2 cell line, in accordance with an earlier report of MGL from *C. tropicalis*, the breast cancer cell line was more sensitive (IC_{50} of 0.13 U/ml) than liver cancer cell line (IC_{50} 0.2U/ml) (Selim *et al*., 2015b). MGL was reported as a potent anticancer agent having IC_{50} values near to the standard drug doxorubicin in various cancer cells with IC_{50} value of 0.127 U/ml (Selim *et al*., 2016), which was once again evidenced through our current study results, hence showing the potential of MGL from the new isolate of *B. haynesii* toward cancer therapeutics.

CONCLUSION

In the current study, among the various isolates screened for MGL production, a new bacteria isolated from the Agumbe forest soil, of the Western Ghats, exhibited the highest specific activity. Based on macro and micro morphological features, the isolate was confirmed as belonging to the genus *Bacillus*. Sequencing of the conserved 16s rRNA region of the organism further confirmed it to be of a new strain of *B. haynesii* named as *B. haynesii* JUB2 and was deposited at GenBank (Sequence ID: NR_157609). The *B. haynesii* family has so far never been investigated for their MGL production, to the best of our knowledge. The crude MGL was subjected to acetone precipitation and DEAE cellulose column purification. The activity of MGL in the 6th fraction of the column purified

sample was 11.07-folds higher than the crude. The biochemical properties of the enzyme with respect to pH and temperature were investigated. The enzyme activity was highest at pH 8.0 at a temperature of 37°C compared to the control of pH 7.0 at a temperature of 28°C. Lyophilized acetone precipitated and column purified fractions were checked for their anticancer potential through a MTT cell viability assay. Our data indicated that significant cytotoxicity was exhibited by the crude enzyme as well as acetone precipitated and column purified fractions on MCF-7 and HepG2 cancer cells, although further studies with higher concentrations and *in-vivo* studies would be necessary. From the current study we can conclude that purified MGL from a new source of *B. haynesii* JUB2 holds promise in anticancer studies. Further research toward complete characterization and statistical optimization of the enzyme production from *B. haynesii* JUB2 in addition to strain improvement studies can be of great pharmacological relevance.

CONFLICT OF INTEREST

Authors declared that there are no conflicts of interest.

FUNDING

None.

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