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Isolation and Characterisation of Thermophilic *Bacillus licheniformis* SUNGC2 as Producer of *α*-Amylase from Malaysian Hot Spring

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ABSTRACT

Screening of new source of novel and industrially useful enzymes is a key research pursuit in enzyme biotechnology. The study aims to report the characteristics of novel thermophilic microorganisms isolated from Sungai Klah (SK) Hot Spring, Perak, Malaysia, that can produce α-amylase. The morphological and biochemical properties were examined for SUNGC2 sample. The isolate was further screened for amylase, followed by 16S rRNA and analytical profile index (API) test. This isolate was further subjected to pH optimisation for *α*-amylase production. It was found that SUNGC2 was an *α*-amylase producer and was identified as *Bacillus licheniformis* SUNGC2 with NCBI accession numbers MH062901. The enzyme was found to exhibit an optimum temperature of 50°C and a pH of 7.0. The relative activity of the enzyme was obtained based on the improvement of the culture conditions. The highest amount of amylase production was 24.65 U/mL at pH 7.0, consecutively the growth was also highest at pH 7.0 with a 9.45-fold increase in specific activity by ammonium phosphate precipitation of 80% (w/v). The results showed that the bacteria isolated from the hot spring are a significant source of thermophilic enzymes that are highly promising in biotechnology.

Keywords: Alpha amylase, *bacillus licheniformis*, thermophilic, hot spring

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INTRODUCTION

The thermophilic *Bacillus* can be isolated from different extreme environments including thermal hot-springs, shallow vents and deep sea hydrothermal with optimum growth temperatures range between 45°C

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and 70°C (Adiguzel et al., 2009). Thermophilic bacilli enzymes can be produced in enormous quantities employing a comparatively less complicated purifying method that is highly beneficial compared to those from mesophilic or psychrophilic bacteria (Burgess et al., 2010). Currently, the screening for a new source of novel and beneficial enzymes for biotech industries is in great demand. Novel enzymes ought to have the benefit of being stable at high temperatures, wide pH range, different concentrations of salts, solvents and also possess a variety of uses in industrial processes. The advantages of using thermophilic enzymes are well documented as industrial catalysts with the potential revenue reflected on the rapid growth of the enzymes market (Burgess et al., 2010; Van Der Maarel et al., 2002). Generally, the extracellular enzymes are more stable at various ranges of temperature and pH, and easier to be isolated and purified compared to the intracellular enzymes (Teodoro & Martins 2000).

The operation of biotechnological techniques at high thermal levels offers several benefits, and one example is temperature increase has a considerable effect on the solubility and bioavailability of organic compounds (Ameri et al., 2015). Consequently, several thermo-active amylases have been isolated and characterised, such as from *Bacillus* sp. (Ardhi et al., 2020), and *Bacillus licheniformis* ATCC 9945a (Božić et al., 2011). Amylase enzymes (*α*-amylase, β-amylase, and γ-amylase) are essential enzymes in current biotechnology and makeup approximately 30% of the global production of enzymes alternating from the process of converting starch to sugar syrups, to producing cyclo-dextrins specifically for pharmaceutics (Van Der Maarel et al., 2002). *α*-amylases are universally allocated all through animals, flora and microbial kingdoms. The production of amylases is economically viable due to the microbial enzyme's ability to be manipulated to obtain enzymes of desired characteristics (Teodoro & Martins 2000). *α*-Amylases cleave α-1,4-glycosidic bonds of carbohydrates and oligosaccharides. Therefore, they are used for industrial starch liquefaction and saccharification (Chai et al., 2016). The amylase family of enzymes is very important because of its versatility as potential industrial applications and among bacteria, *B. licheniformis, B. subtilis, B. stearothermophilus* and *B. amyloliquefaciens* have been extensively commercialised and applied for the production of the enzymes for different uses (Deljou & Arezi 2016; Vidyalakshmi et al., 2009). Their applications in biotechnology include starch processing, biofuel, food, paper, textile and detergent industries, bioremediation of environmental pollutants and in clinical and medical applications. Industrial production of enzymes requires high productivity and in certain condition, the application of wild-type strains are not suitable for enzyme production (Jujjavarapu & Dhagat 2019). The properties of α-amylases such as thermostability and pH profile should match its application. Therefore, the diversity of the applications creates the need to search for novel *α*- amylases with advanced and enriched properties (Panosyan et al., 2020). The raw starch degrading amylases ideal for use in industry as well as for their attractive production cost-effectiveness (Mohammad et al., 2017).

Hot springs are promising environments for thermophilic microorganisms and in the past few decades, hot springs globally have proven to be significant targets to isolate new thermotolerant or thermophilic microorganisms as a source for the production of thermozymes that offer significant stability (Verma et al., 2014). Malaysia has a significant number of hot springs, especially along the edge of the Banjaran Titiwangsa mountain range (Samsudin et al., 1997). The Sungai Klah (SK) Hot Spring, Perak ranks as the second hottest geothermal spring in the country. It has increased in attractiveness as it is considered a natural biomass degrading bioreactor because of the existence of an underwater vegetation bed (Chan et al., 2015; Lee et al., 2018). Biological studies of the Malaysian hot springs are limited due to the lack of complete information on their microbial communities (Chan et al., 2017). Nonetheless, (SK) Hot Spring has an abundant and varied phylogenetic population of thermophiles and a source with potential to isolate bacteria capable of producing amylase. This is due to its natural environment, high total organic carbon (TOC), plant litter shallow stream and geochemical parameters and a wide range of temperature and pH (Chan et al., 2017; Msarah et al., 2018). Nevertheless, no persistent research has focused on further utilisation of these thermophiles. The current study aims at amylolytic screening, isolation and characterisation of new thermophilic microorganisms isolated from Sungai Klah Hot Spring, that can possess high biotechnological and environmental potential, and as a continuous line of research for thermophilic bacteria acquired from hot springs in Malaysia.

MATERIALS AND METHODS

Isolation, Cultivation and Qualitative Screening of *α*- Amylase Producer Bacteria

The strains used in this study have been isolated from Sungai Klah Hot Spring, Perak, Malaysia. The 23 samples isolated were then serially diluted from 10^{-1} to 10^{-6} with sterile distilled water and spread on nutrient agar (NA) plates. The inoculated plates were incubated at 50°C for three days with morphological observation of the culture. The shape, size, colour, elevation and margin of the colonies were identified and the pure colonies were streaked on a NA containing 1% starch (Starch NA), incubated and observed for 36 hours at 50°C. Single colonies confirm as amylase production by forming clear hydrolysis zones after a 0.5% (w/v) iodine solution was dispensed over the isolates.

Identification, DNA Sequencing and Phylogenetic Analysis

Bacterial isolates were investigated for its physiological, cultural, morphological and biochemical characteristics (Harley & Prescott 2005). Biochemical tests for the identification of thermophilic isolates including the production of indole, catalase, citrate and oxidase, urease test, and nitrate reduction were carried out. Colony grown on NA was used for the determination of colony morphologies. The growth temperature range was confirmed through incubation of the isolate at 30, 40, 50, 60 and 70°C. Bacterial growth

on NaCl (1% to 7% (w/v)) and on blood agar were also observed. Further, isolates were identified using Analytical Profiling Index (API) strip tests according to the manufacturer's instructions for API 50 CHB and API20E strips (bioMérieux, SA, Marcy-l'Etoile, France). Bacterial suspension (100 µL) was inoculated into the strips and incubated in a temperature ranged from 50-55°C for 48 hours. The pattern of the reactions obtained was coded into a numerical profile.

16S rRNA gene sequence analysis was conducted to confirm the identification of bacterial isolates. Amplification of the 16S rRNA gene was carried out by polymerase chain reaction (PCR) with Prime Thermal Cycler (Techne®/Bibby Scientific, UK), using forward primer, 8-27 F, 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer, 1492 R, 5'- GGTTACCTTGTTACGACT T-3'.

The bacterial genomic DNA of the isolates was subjected to purification employing the TE boil extraction method which is the modified protocol for bacterial DNA extraction (Li et al., 2003). Bacterial culture was grown in nutrient broth at 50°C for 18 hours. An appropriate number of bacterial cells was transferred to 1.5 mL micro-centrifuge tube and it was centrifuged for 1 min at 11200 x g. The pellet was suspended in 200 µL TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA], and the mixture was briefly vortexed. The suspension was then put in a boiling water bath at 100°C for 1 min, then freezed at -70°C for 3 min. The next step involved heating in boiling water bath at 100°C for 2 min, freezing at -70°C for 3 min (repeating for two times), then finally subjecting to centrifugation for 5 min at 11200 x g. The supernatant (100 µL) was placed into a sterile tube and kept at -20°C for PCR. PCR was conducted using PROMEGA Go Taq®/USA Green Mix, 2X. PCR protocol was subjected to heating up to the temperature of 94°C for 5 min for the initial DNA denaturation, and then by 30 cycles with the following cycling profile: 94°C for 1 min, 54°C for 1 min, and 1.5 min at 72°C for annealing. A final extension step was conducted following the amplifying reaction for 5 min at a temperature of 72°C. The range of identity for identifying bacteria employing the 16S rRNA gene analysis is ≤ 99% and ≥ 97% to the GenBank database and according to Drancourt et al. (2000) it permits distinguishing the strain at the genus level.

Bacterial Isolate *α*- Amylase Production Medium

Bacterial isolate was investigated for α-amylase production on a medium (g/L): NaCl (0.1), soluble starch (10), magnesium sulphate $MgSO_4.7H_2O$ (1.0), disodium phosphate Na_2HPO_4 (3.0), peptone (2.0) and ferrous sulphate $FeSO_4$ (0.03). Erlenmeyer flask (250 mL) consisted of 1 mL inoculum ($1x10^8$ cells/mL) mixed with 99 mL cultivation medium was incubated in an orbital shaker at 50°C/48 hours and agitated at 100 rpm. Filtration of the medium was done by using Whatman No.1 filter paper every 6 hours intervals. The cell free filtrate was employed for the *α*-amylase assay and the bacterial growth was measured at 600 nm (Kumar & Raja, 2019). The protein level was decided based on the approach

described by Bradford (1976) at 595 nm. The total protein was expressed as mg/mL. All experiments were carried out in triplicates in at least three different occasions.

Amylase Assay

The *α*-amylase activity was carried out by using a modification of the di-nitro-salicylic acid (DNS) technique on the basis of the reducing sugars freed from the soluble starch and were determined by glucose standard curve (Miller, 1959). The standard curve was prepared by dissolving 100 mg of glucose in 100 mL of distilled water and working standard was prepared by diluting 10 mL of stock solution to 100 mL with distilled water. The standard curve was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard glucose solution and the final volume was made up to 1 mL by adding distilled water. One mL of glucose oxidase peroxidase reagent was added and the mixture was incubated at 35 ºC for 40 min. The reaction was terminated by the addition of 2 mL of 6N-HCl, and the absorbance was recorded at a wavelength of 540 nm using Spectrophotometer. One mL soluble starch (1% soluble starch (w/v) in sodium citrate buffer (0.05 M/ pH 5.9) was heated in a water bath at 50°C for 10 min. Then, 0.1 mL of crude enzyme was added to the substrate, followed by incubation at 50°C for 10 min through mild shaken. The reaction was halted by adding 2.0 mL of DNS reagent. The reaction mixture was then subjected to heat for 10 min at 100°C, then allowed to cool to 27°C before dilution with distilled water (16.9 mL). Then the mixture measured by using UV-Visible spectrophotometer at 540 nm absorbance and the specific activity was then calculated and and reported in units per millilitre (U/mL). The enzyme acitivity is calculated by measuring umole of product formed (glucose) in 10 minutes assay by 0.1 mL enzyme sample (which will give umole/ min/mL) (Ibrahim et al., 2013).

Optimisation of pH

The optimisation of culture conditions was evaluated with a variation of pH (ranging between 3.0, 7.0 and 9.0). The incubation temperature was set at 50°C, the agitation speeds of 200 rpm, and the inoculum size of 0.5 McFarland to increase amylase production by *B. licheniformis* SUNGC2. The medium was filtered using Whatman No.1 filter paper after 18 hours of cultivation and then the cell free filtrate used for the amylase assay. The mixture's absorbance was verified at 540 nm and bacterial growth was measured at 600 nm absorbance. All experiments were carried out in triplicates for at least three different occasions (Ardh et al., 2020).

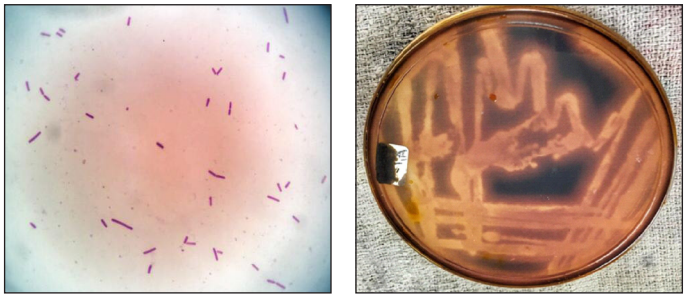
RESULTS AND DISCUSSION

Isolation of *α*-Amylase Producing Bacteria

The knowledge about thermophilic bacteria and the enzymes they produce from hot springs in Malaysia is significant and still moving forward in comparison with additional

hot springs located worldwide (Chan et al., 2015, 2017; Msarah et al., 2018, 2020). In this study thermophilic α-amylase producing bacteria were positively isolated from Sungai Klah Hot Spring in Perak, Malaysia. α-Amylase activity was confirmed by the appearance of a clear surrounding (halo) of the colonies after staining with Lugol's iodine (Figure 1b). The qualitative screening of 23 isolates showed that isolate SUNGC2 produced the largest hydrolysis zone and therefore was selected for further optimisation process.

Enzyme production primarily linked to the growth of the microorganisms is called growth associated-enzymes, and according to Niu et al. (2009) and Asoodeh et al. (2010) some starch degrading enzymes such as α-amylases are produced according to this mechanism. The results displayed that enzyme production was related to the growth of the isolate SUNGC2 (0.261 mg/mL) at 18 hours (Figure 2). SUNGC2 showed a decline



(a) (b)

Figure 1. Microscope (a) and amylase qualitative screening (b) of *B. licheniformis* SUNGC2

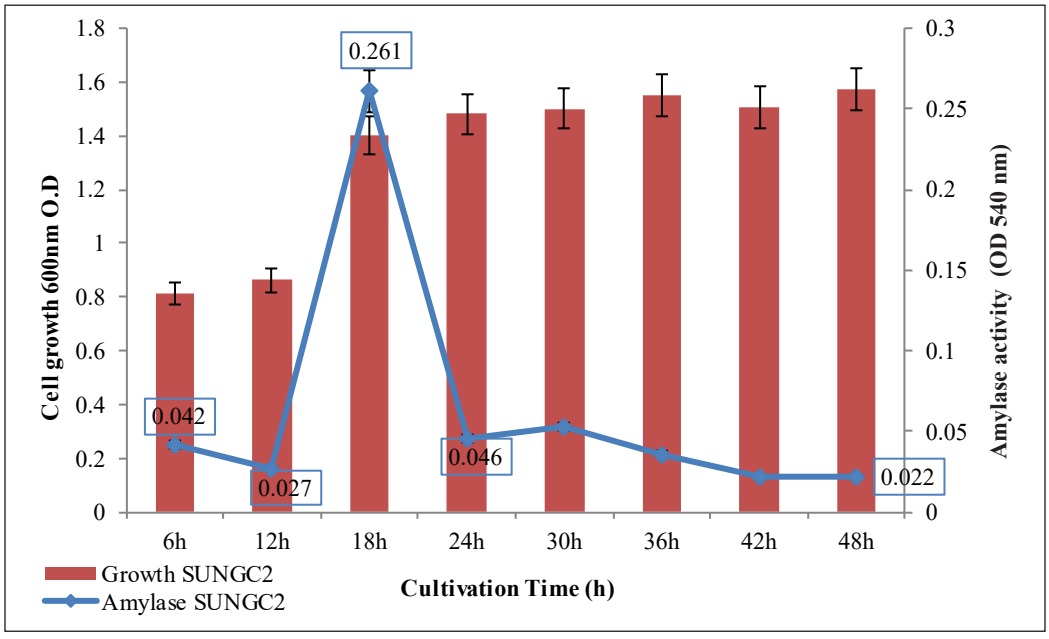


Figure 2. Amylase production and growth profile of *B. licheniformis* SUNGC2

in amylase production after 18 hours until it reached 0.022 mg/mL at 48 hours, while the growth of SUNGC2 was increasing in log phase.

Identification of *α*-Amylase Producer Isolate SUNGC2

SUNGC2 was identified based on its biochemical tests and microscopic characteristics. SUNGC2 was a Gram positive, rod-shaped bacterium, a pale colony in colour, flat elevation with irregular margins and producing distinctive scent. Additionally, the biochemical and cultural characteristics were also observed for further identification (Table 1). SUNGC2 showed positive results for the catalase production, nitrate reduction, citrate tests, grew well in 7% sodium chloride medium and positive beta hemolytic on blood agar. However, urease utilisation and indole tests showed negative results. It was observed that SUNGC2 grew at temperature of 55°C. SUNGC2 was identified as a *Bacillus* sp. based on 16S rRNA identification. The *Bacillus* sp. together with the thermophilic bacilli in general require uncomplicated nutritional requirements; hence, they have no requirement for any particular amino acids for growth and are able grow on enriched media like tryptone soya agar (TSA) or nutrient agar (NA) (Haki & Rakshit 2003). Thermophilic *Bacillus* sp. possess an optimum growth temperature typically ranging from 50 to 70°C but differ among species and strains. They can be readily cultured and sub-cultured in the laboratory and utilised to extract useful compounds (Burgess et al., 2010).

Table 1
Microscopic, morphology and biochemical characteristics of B. licheniformis *SUNGC2*

Characteristics	SUNGC2	Biochemical tests	SUNGC2
Colony	Irregular	Indole production	Negative
Margin	Irregular	Catalase production	Positive
Elevation	Flat	Citrate production	Positive
Surface	Shiny and moist	Oxidase production	Negative
Color	Pale	Urease test	Negative
Odor	Yes	Nitrate reduction test	Positive
Growth	Aerobic and facultative anaerobic growth	Growth at 55°C	Positive
Gram staining	Positive	Hemolysis on the blood agar plate	β-hemolytic
The shape of vegetative cells	Rod-shaped	Growth in 7% sodium chloride	Positive

Gene analysis was achieved by using 16S rRNA amplification and 301 bases nucleotide sequence was obtained. Sequence then was analysed using Basic Local Alignment Search Tool (BLAST). The results showed 98% similarity with *Bacillus licheniformis*. The sequences of SUNGC2 were stored in the GenBank database according to accession numbers of MH062901. The phylogenetic tree was built based on 16S rDNA sequence

alignment employing Neighbor-Joining technique for the SUNGC2 strain (Figure 3). A total of 235 positions can be found in the final dataset. Evolutionary analyses were carried out via MEGA7 software (Kumar et al., 2016). Therefore, it was recommended that isolate SUNGC2 identified as *B. licheniformis* with the strain name of SUNGC2. Additional identification was then performed by API CHB50. SUNGC2 was identified by the API 50 CHB identification kit to provide additional details of the isolate's metabolic abilities.

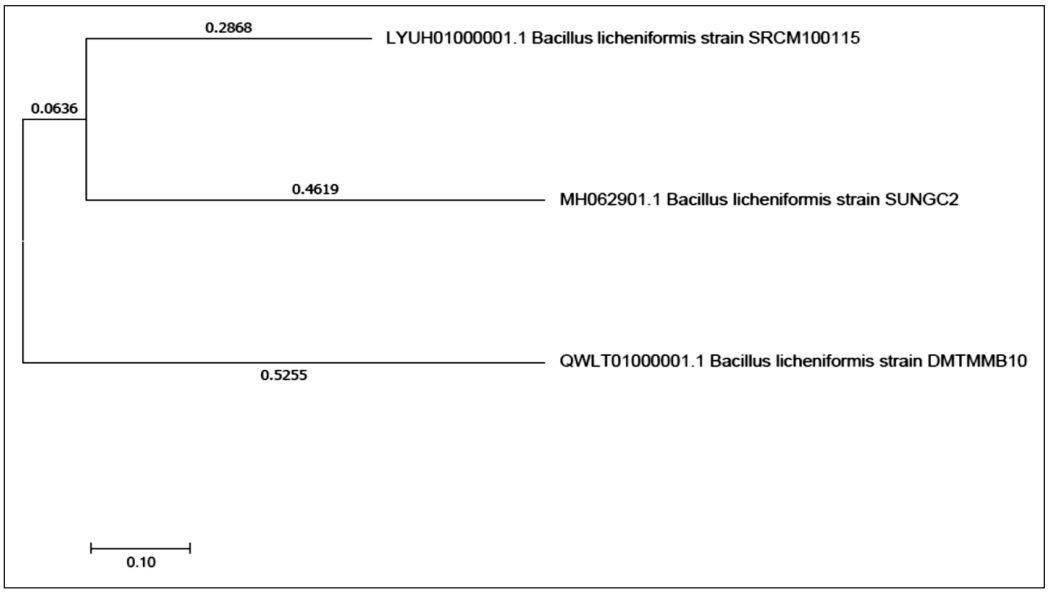


Figure 3. Evolutionary relationships of taxa for *B. licheniformis* SUNGC2

The outcomes are presented in Table 2 and Table 3. The isolate was identified as *B. licheniformis* according to the combination of partial 16S sequencing analysis and API 50CHB.

The API 50 CHB and API 20E result using apiweb™, showed a very good agreement with the previous results, that strain SUNGC2 is *Bacillus licheniformis* with % ID of 99.3 homology.

Table 2
API50CH profile of B. licheniformis *SUNGC2*

Test No.	Substrate	SUNGC2	Test No.	Substrate	SUNGC2
0	Control	-	26	Salicin	+
1	Glycerol	+	27	D-Cellobiose	+
2	Erythritol	+	28	D-Maltose	+
3	D-arabinose	+	28	D-Lactose	+
4	L-arabinose	+	29	D-Sucrose	-
5	Ribose	+	30	Trehalose	-

Table 2 *(continue)*

Test No.	Substrate	SUNGC2	Test No.	Substrate	SUNGC2
6	D-xylose	+	31	Gentiobiose	+
7	L-xylose	-	32	Melibiose	+
8	Adonitol	-	33	Raffinose	+
9	ß methyl-D-Xyloside	-	34	Melezitose	-
10	Galactose	+	35	Starch	+
11	Glucose	+	36	Glycogen	+
12	Fructose	+	37	Inulin	+
13	Mannose	+	38	D-Turanose	-
14	L-Sorbose	-	39	D-Tagatose	+
15	Rhamnose	+	40	D-Fucose	+
16	Dulcitol	-	41	L-fucose	-
17	Inositol	+	42	D-Lyxose	+
18	Sorbitol	+	43	D-Arabitol	-
19	Mannitol	+	44	L-Arabitol	-
20	L-Methyl-D-mannoside	-	45	L-Sorbose	-
21	D-Methyl-D-glucoside	+	46	Xylitol	-
22	N-Acetylglucosamine	+	47	Gluconate	+
23	Amygdalin	+	48	2-Ketogluconate	-
24	Arbutin	+	49	5-Ketogluconate	-
25	Aesculin	+			
Significant taxa	*Bacillus licheniformis*	% ID 99.3	T		

+, positive reaction; - negative reaction; ? non conclusive

Table 3
API 20E profile of B. licheniformis *SUNGC2*

Strains code	API 20E														
SUNGC2	**ONPG**	**ADH**	**LDC**	**ODC**	**Citrate**	**H_2S**	**Urease**	**TDA**	**Indole**	**VP**	**Gelatin GEL**	**Nitrate NIT**	**Temperature**	**Incubation**
	+	-	-	-	+	-	-	-	-	+	+	+	45h	24
		1			2			0			7			h

pH Optimisation for *α*-Amylase Production

In our study, an additional rise in the pH level led to a reduction in the activity of α-amylase as observed by Teodoro and Martins (2000). Figure 4, the amylase production was maximum at pH 7.0 (24.65 U/mL) and minimum at pH 9.0 (7.65 U/mL). The highest specific activity

obtained by ammonium phosphate precipitation 80% (w/v) was 3.79 U/µg for SUNGC2 (9.45-fold increase). Various researches have mentioned that the optimisation of amylase production by *Bacillus* spp. is due to the specific enzyme conditions for each application (Hmidet et al., 2009). Commonly, the enhancement of the microbial production of enzymes involves the optimisation of environmental parameters such as temperature, pH, and nutrients. Amylase stability is beneficial for various applications and the characterisation of enzymes is significantly important for industrial applications. In this study, the pH effects on amylase activity in a range of 3.0-9.0 and are represented in Figure 4.

Enzymes from thermophilic microorganisms have special characteristics such as high stability to changes in wide range of pH (Alrumman et al., 2018). Elkhalil and Gaffar (2011) reported that the pH activity profile of α-amylase produced from *B. acidocaldarius* had an activity optimum at pH 6.0. Several researchers studied the production of α-amylase from *Bacillus* sp. and found its maximum activity at pH between 5.0 and 6.5 (Ardhi et al., 2020; Deljou & Arezi 2016; Teodoro & Martins 2000). However, Thippeswamy et al. (2006) reported a pH of 6.5 as an optimum for *α*-amylase activity. It was detected that the highest amylase activity occurred at pH 6.0. However, the highest bacterial growth occurred at pH 8.0, suggesting that the bacteria need an alkaline environment to synthesise the enzymes and that synthesis was not growth dependent.

Similarly, the growth was highest at pH 7.0 and had a proportional relationship to the amylase production. The amylase extracted from *B. licheniformis* SUNGC2 has wide-

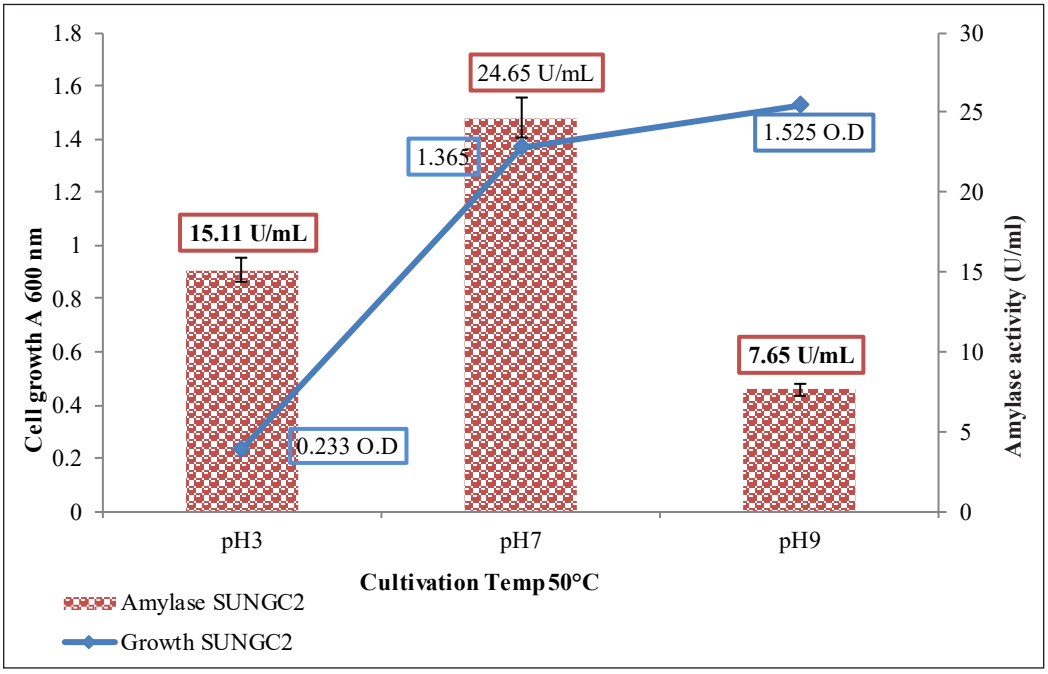


Figure 4. Effect of growth and pH on the enzyme activity of *B. licheniformis* SUNGC2

ranging pH activity (pH 3.0-9.0) with optimal pH at 7.0 which is close by to the optimum pH value of most *Bacillus* sp. amylase (Divakaran et al., 2011). The enzyme had about 30% relative activity at pH 9.0 and about 50% at pH 3.0. At pH 7.0, the enzyme expressed 100% relative activity. According to the results, the amylase activity of *B. licheniformis* SUNGC2 was observed to be maximum at pH 7.0 which in agreement to the findings by Oyeleke and Oduwole (2009) and Vidyalakshmi et al., (2009). Nevertheless, other research on amylase activity of *B. licheniformis* MIR 29 was found to be optimum at pH 9.0 (Ferrero et al., 2009).

CONCLUSIONS

The thermophilic *B. licheniformis* SUNGC2 was isolated and characterised from a Sungai Klah Hot Spring water samples from Perak, Malaysia. SUNGC2 was able to produce *α*-amylase at 50°C and the growth and enzyme activity were showed to be optimum at pH 7.0. At 18 hours of cultivation at pH 7.0, the *α*-amylase activity was shown to be increased by 9.45-fold compared to control. The results showed promising thermophilic microorganism *B. licheniformis* SUNGC2 capable of producing thermostable *α*-amylase, with stability over wide-ranging pH which makes SUNGC2 a good candidate for various applications in biotechnology.

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