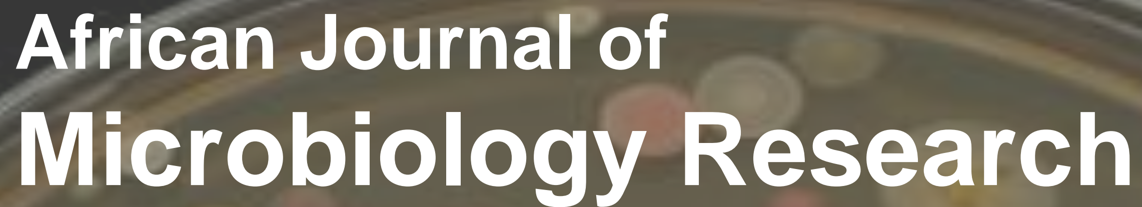

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African Journal of Microbiology Research

Full Length Research Paper

Degradation of diesel-oil by a newly isolated *Kocuria sediminis* DDK6

Ashraf Y. Z. Khalifa

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A bacterial strain named DDK6 was isolated from diesel-contaminated soil from a petrol station in Al-Hofuf city, after enrichment on diesel oil. The strain DDK6 formed a reddish-pink colony with a 2 to 3 mm diameter after two days of incubation at 30°C. Cells were Gram-positive coccoid and formed no endospores. Phenotypic identification by the automated bacterial system, Vitek compact II, identified the DDK6 as *Kocuria* sp. at 95% probability level. The 16s rRNA gene sequencing analysis confirmed the identity of the strain as *K. sedimins* at an identity level of 99.15%. Results of Gas chromatography-mass spectrometry (GC-MS) revealed that the DDK6 degraded the C14-C19 compounds in diesel. In addition, the DDK6 strain consumed the majority (68%) of the carbon sources tested, including monosaccharides, disaccharides, polysaccharides, and sugar alcohols as noticed by biochemical characterization using the API 50CH. The cultural, biochemical, and molecular characteristics were in general agreement with the strain identification. The results confirmed the metabolic versatility of the strain DDK6, in addition to its ability to degrade diesel oil, thereby providing ecological and environmental merits for its application in bioremediation of hydrocarbon pollutants.

Key words: Bioremediation, hydrocarbon, *Kocuria*, diesel oil.

INTRODUCTION

There is an increasing concern globally about potential environmental consequences arising from contamination by accidental petroleum release during storage, transport, or exportation. Saudi Arabia is one of the largest oil-producing countries worldwide, therefore marine and terrestrial biota are negatively affected. Diesel oil is a common fuel for diesel engines. Chemically, it is a mixture of aliphatic and aromatic hydrocarbons produced during petroleum separation by fractional distillation. Leakages of diesel oil can occur from storage tanks when the oil seeps into soils and groundwater causing severe environmental problems (Das and Chandran, 2010). The ecological effects of diesel on plants growing on diesel-contaminated soils result in reduction of seed germination,

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plant growth and productivity. Additionally, diesel sticks with soil particles causing bad soil aeration, leading to a decline in microbial biodiversity. Consequently, the ecosystem functioning is negatively affected and the biogeochemical cycling of essential elements become truncated (Ciric et al., 2010).

One promising and efficient strategy to remove diesel oil from soil and water is via microbial degradation. Bioremediation is an ecofriendly, cost-cheap, versatile and efficient treatment of hydrocarbons. It has been confirmed in many laboratories worldwide that several bacterial groups possess the catabolic machinery for diesel oil. Bacterial species, which belong to *Acinetobacter, Bacillus, Citrobacter, Corynebacterium, Flavobacterium* and *Micrococcus* are representatives of oil-degrading bacteria (Jirasripongpun, 2002; Das and Chandran, 2010). Furthermore, hydrocarbon-degrading bacteria that have recently been documented in different localities in Saudi Arabia, include *Stenotrophomonas maltophilia* (Arulazhagan et al., 2017), *Cupriavidus taiwanensis*, *Ochrobactrum intermedium*, *Pseudomonas aeruginosa* and *P. citronellolis*, (Oyehan and Al-Thukair 2017).

Kocuria is Gram-positive cocci arranged in pairs, short chains, tetrads, cubical packets of eight and irregular clusters. *Kocuria* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, order *Actinomycetales*, sub order *Micrococcinae* and family *Micrococcaceae*. The genus *Kocuria* was coined by Stackebrandt et al. (1995) and separated from *Micrococcus* based on chemotaxonomic and phylogenetic features (Stackebrandt et al., 1995). Currently, there are more than 18 species of *Kocuria* identified based on the 16S rRNA phylogenetic studies. In general, *Kocuria* spp. are non-pathogenic however, some species have been isolated from infected superficial and deep human tissues. At the time of writing, 20 different species with validated names are included under the genus *Kocuria.* The completely detailed list of the species is outlined in http://www.bacterio.cict.fr/k/kocuria.html. Most of these bacterial species have been isolated from diverse ecological niches. For example, *K. dechangensis* obtained from saline and alkaline soils (Wang et al., 2015), *K. salsicia* from salt-fermented seafood (Yun et al., 2011), *K. gwangalliensis* from seawater (Seo et al., 2009), *K. palustris* and *K. rhizophila* from rhizoplane of *Typha angustifolia* (Kovács et al., 1999), *K. pelophila rhizosphere* of a mangrove (Hamada et al., 2016). Additionally, *K. sedimins* has been isolated from a sediment sample from Kerala, India and described by polyphasic approaches (Bala et al., 2012) but its ability to degrade diesel oil was not investigated. Researchers have showed the role of *Kocuria* spp. in bioremediation of hydrocarbon (Esmaeil et al., 2009), removal of copper from copper- contaminated soils (Achal et al., 2011) and production of probiotics (Sharifuzzaman et al., 2014), biocontrol agents (Sharifuzzaman and Austin 2010), plant-growth-promoting activities (Egamberdieva, 2008). Members of the genus *Kocuria* have been shown to produce, kocurin, a novel thiazolyl peptide antibiotic, which exhibited anti-bacterial activities against clinically relevant strains (*Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii* and *Candida albicans*) (Palomo et al., 2013).

Hydrocarbon-degrading bacteria that have recently been investigated in different localities in Saudi Arabia, include *Stenotrophomonas maltophilia* (Arulazhagan et al., 2017),

Cupriavidus taiwanensis, *Ochrobactrum intermedium*, *Pseudomonas aeruginosa* and *P. citronellolis*, (Oyehan and Al-Thukair 2017). Jeddah, Khobar and Duhran. However, little is known about the biodegradability of diesel-oil in Al-Houf, eastern region, Saudi Arabia. In addition, the search for local diesel-oil-degrading bacteria from soils exposed to the prevailing conditions in Al-Houf city has a pivotal importance in bioremediation approaches. Therefore, the current study aimed at isolation and characterization of a bacterial strain, designated DDK6, which was able to grow on diesel as a sole energy and carbon sources. To fulfill this aim, a soil sample was collected from a diesel-contaminated soil from a petrol station.

MATERIALS AND METHODS

Collection of soil sample

A diesel contaminated soil sample was collected a petrol station in Al-Hofuf Saudi Arabia, in a sterilized screw-capped test tube. In the laboratory, 0.5 g of soil sample was enriched with diesel oil (1%v/v) as a sole carbon source in a 200 ml conical flask containing 50 ml mineral salts (MS) medium with the composition; 1 g $(NH4)_2SO_4$, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, and 5 mg $FeSO_4{\cdot}7H2O$ in 1 L distilled water, pH 7) (Wu et al., 2013). Flasks were placed in a shaking-incubator at 100 rpm for three weeks. To ensure oil degradation ability, subsequent transfer of inoculum (3%) into a fresh MS medium was carried out.

Isolation, purification and preservation of the strain

Aliquots (100 µl) from the diesel-enriched media were streaked into MS agar plates and sprayed with diesel on the surface. Plates were incubated at 30°C for 7 days. Single colonies were picked and sub-cultured into fresh MS agar amended with diesel.

Morphology of colony and cells

The morphological characteristics of the colony; pigmentation, diameter, elevation and transparency was determined visually of 24 h old colony growing on soya agar medium and incubated at 30°C. Cell shape, arrangement and reaction to the gram staining were assessed.

Table 1. Cultural and cellular characteristics of the strain DDK6.

Feature	Description
Size	2 mm
Shape	Circular
Pigmentation	Reddish pink
Texture	Smooth
Elevation	Convex
Edge	Entire
Cells	Coccoid
Gram Reaction	Positive
Endospore formation	None

Biochemical characterization using the API50Ch strip kit

In order to investigate the biochemical characteristics of the strain DDK6 API50Ch strip kit (Biomerieux, France) was used following the guidelines of the manufacturer. Results were recorded after 48 h of incubation of API50Ch.

Identification of the strain using Vitek compact II

The bacterial strain DDK6 was identified using the automated system for identification of bacterial strains, Vitek compact II. Gram-Positive Card was used and the data analysis were carried out using the Software version: 05.02 (Biomerieux, Mary L'Etoile, France).

Identification of the strain DDK6 using 16S rRNA gene sequencing

PCR amplification of the 16S rRNA gene

16S rRNA gene was amplified using the universal primers; 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3' (Weisburg et al., 1991) in 20 µl total PCR reaction. Genomic DNA was extracted from the strain using the InstaGene Matrix (Bio-Rad, USA) following the instructions of the manufacturer. PCR conditions were adjusted as previously described (Khalifa et al., 2015).

16S rDNA sequencing and construction of phylogenetic tree

For sequencing of the 16S rRNA gene, the Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) was used. Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Blast search and calculations of similarity values of pairwise nucleotide 16s rRNA gene sequence were performed using EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). Multiple alignments with sequences of the most closely related recognized *Kocuria* strains and construction of phylogenetic tree were carried out by using the Neighbor-joining method based on the Tamura-Nei model (Tamura and Nei, 1993). Evolutionary analyses were conducted in MEGA5.02 (Tamura et al., 2011). The 16s rRNA gene sequence of strain DDK6 was deposited in the NCBI database under the accession number KY307788.

Detection of diesel oil degradation using the Gas chromatography-mass spectrometry (GC-MC)

The ability of the strain DDK6 to degrade diesel oil was further detected using GC-MS. DDK6 was inoculated into 50 ml MS medium containing 1% (V/V) diesel oil and incubated at 30°C with shaking 150 rpm for 5 days. Non-inoculated flask containing the same growth medium was used as control. After incubation period, diesel oil was extracted using equal volume of dichloromethane and analyzed using a Shimadzu GCMS –QP2010 SE instrument. The flow rate of the Helium as a carrier gas was set at 6.0 ml/min. The temperatures of injector and detector were adjusted at 250 and 300°C, respectively. The temperature program was as followings: 2-min hold at 60°C, ramp to 300°C at 20°C/min and 6-min hold at 300°C.

RESULTS AND DISCUSSION

K. sediminis strain DDK6 was isolated from diesel-contaminated soils from a petrol station in Al-Hofuf. The cultural characteristics are outlined in Table 1. DDK6 formed a reddish-pink, convex colony, with a diameter of 2 to 3 mm, and entire margin. Cells of the strain DDK6 were coccoid, formed no endospores and exhibited positive reaction to the Gram staining. These features were typical to the type strain of *K. sediminis* FCS-11^T (Bala et al., 2012) confirming the identity of the DDK6 strain.

Biochemical characterization using the API50Ch strip kit

The biochemical characteristics of the strain DDK6 using the API50Ch (Biomerieux, France) were shown in Table 2. API 50 CH is a phenotypic-based system, comprising 50 biochemical tests designated to estimate the carbohydrate metabolism ability of a microorganism. The system is exploited in the current study as a fast, effective a reliable tool to study the metabolic versatility of the strain DDK6 towards different sources of carbohydrate.

Table 2. API 50Ch for bacterial strain DDK6.

Number	Test	Result
0	Control	-
1	Glycerol	+
2	Erythritol	-
3	D-arabinose	+
4	L-arabinose	+
5	D-ribose	+
6	D-xylose	+
7	L-xylose	+
8	D-adonitol	+
9	Methyl-BD-xylopyranoside	+
10	D-galactose	+
11	D-glucose	+
12	D-fructose	+
13	D-mannose	+
14	L-sorbose	+
15	L-rhamnose	-
16	Dulcitol	+
17	Inositol	+
18	D-mannitol	+
19	D-sorbitol	+
20	methyl-αD-mannopyranoside	+
21	Methyl-aαD-glucopyranoside	+
22	N-acetylglucosamine	+
23	Amygdalin	+
24	Arbutin	+
25	Esculin	+
26	Salicin	+
27	D-cellobiose	+
28	D-maltose	+
29	D-lactose	+
30	D-melibiose	+
31	D-saccharose	+
32	D-trehalose	+
33	Inulin	-
34	D-melezitose	-
35	D-rafinose	-
36	Starch	+
37	Glycogen	+
38	Xylitol	-
39	Gentibiose	+
40	D-turanose	-
41	D-lyxose	-
42	D-Tagatose	-
43	D-Fucose	-
44	L-Fucose	-
45	D-arabitol	-
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48	potassium 2- ketogluconate	-
49	potassium 5-ketogluconate	-

DDK6 exhibited the ability to utilize a wide range of different carbon including monosaccharides (e.g., D-galactose and L-arabinose) disaccharides (e.g., D-maltose and D-lactose), polysaccharides (e.g., Starch and Glycogen) and sugar alcohols (e.g., D-galactose, D-mannitol). Out of 49, DDK6was able to utilize 33 (~67%) different carbon compounds tested. However, 33% of the carbon compounds were not metabolized by the strain under study. D-turanose, D-lyxose, D-Tagatose, D-Fucose, Arabitol, potassium gluconate and Inulin are representatives of carbon sources that were not consumed by the strain DDK6. The biochemical profile of the strain DDK6 is shown by the API 50CH kit strip is typical to that of the *K. sediminis* (Bala et al., 2012) providing another evidence to the reliable identification of the strain DDK6. Genome sequences of members of *Kocuria* genus such as *K. marina* SO9-6 (Castro et al., 2015) and *K. rhizophila* strain TPW45 (Tan et al., 2016) revealed the existence of many gene clusters involved in catabolic pathways of carbohydrate. The ability of the strain DDK6 to grow using a wide range of different biochemical compounds. This range of metabolic versatility could provide an explanation about the ubiquity of *Kocuria* sp. in diverse ecological niches particularly, in oil-contaminated ecosystems.

Identification of the strain using mass spectrometry technology

Vitek II is a powerful and accurate automated platform with an expanded identification database for rapid microbial identification, and antibiotic susceptibility testing based on biochemical analysis using colorimetry. The bacterial strain DDK6 was identified as *Kocuria* sp. (at 98%) by using the Vitek compact II indicating that this tool is efficient for bacterial identification at the genus and specific levels. The efficiency of this system to correctly identify *Staphylococcus* spp. and other bacterial genera has been proven previously (Chatzigeorgiou et al., 2011; Paim et al., 2014). Nonetheless, discordance between VitekII and 16S rRNA gene sequencing for bacterial identification has been reported. For example, VitekII identified a bacterial strain from human blood as *K. kristinae* (score of 98%) whereas 16s rRNA sequencing identified it as *Rothia amarae* (Abouseada et al., 2016).

16S rDNA sequencing and of phylogenetic analysis

The 16S rRNA gene sequencing is a well-established technique for a robust and accurate bacterial identification and for inferring phylogenetic relationships among species. Comparative analyses of the 16S rRNA gene sequencing revealed that the strain DDK6 was related to the genus *Kocuria*. Sequence analysis showed that DDK6 was most closely related to *K. sediminis* strain FCS-11^{T} (99.15% identity), followed by *K. flava* HO-9041^{T} (98.9%), *K. turfanensis* HO-9042^{T} (98.9%), *K. dechangensis* NEAU-ST5-33^{T} (98.41%), *K. polaris* CMS 76or^{T} (98.28%), *K. rosea* DSM 20447^{T} (98.27%), *K. aegyptia* YIM 70003^{T} (98.28%), *K. himachalensis* K07-05 ^{T} (97.51%), *K. atrinae* P30 ^{T} (97.1%) at similarity levels of gene sequences. As can be seen in Figure 1, the neighbour-joining phylogenetic tree (Figure 1) clearly highlighted that the strain DDK6 grouped with the *K. sediminis* strain FCS-11. The 16S rRNA gene sequencing confirmed phenotypic identification of the strain DDK6 based on Vitek II. Similar results have been obtained by Hassan et al. (2016) who showed that both Vitek2 and 16S rRNA gene sequencing correctly identified *K. kristinae* (99%) (Hassan et al., 2016).

As can be seen in Figure 2, the major components of the diesel oil were C14-C19 alkanes. The peak areas of the C14-C19 were significantly lower than those in the control indicating that DDK6 was able to degrade this fraction of diesel oil (Figure 2). Our reseults are in accordance with those obtained by Mariano et al. (2007) who demonstrated that *K. palustris* was able to efficiently degrade diesel oil (Mariano et al., 2007). Microbial biodegradation of diesel oil and/or its components is a common process in terrestrial and aquatic ecosystems (Austin and Groves et al., 2011). Two main classes of metalloenzymes are involved in this multi-step process. The first class comprises membrane-associated enzymes such as alkane hydroxylase and the latter composed of cytoplasmic –soluble enzymes such as cytochrome P540. The existence of the alkane hydroxylase and cytochrome P540 in the strain DDK6 is consistent with previous studies on other bacterial species such as *Acinetobacter* sp. (Hou et al., 2013), *Enterobacter cloaecea* (Ramasamy et al., 2017), *Pseudomonas aeruginosa* and *Bacillus subtilis* (Safdari et al., 2017). Both enzymes exhibited wide range of alkane substrates and could have synergistic effect. The ability of other Kocurial species to grow on oil and other hydrocarbon as a sole carbon and energy sources has been documented. For example, *K. flava* and *K. rosea* were shown to degrade naphthalene, phenanthrene and fluoranthene and crude oil (Tumaikina et al., 2008). Apparently, the efficiency of local bacterial strains in hydrocarbon-degradability was found to be substantially higher than that of the introduced strains (Wu et al., 2013), this could be attributed to outcompeting, biotic and/or abiotic interacting factors. Therefore, isolation of new bacterial strains adapted to the local conditions of a particular area is crucial for the efficiency of hydrocarbon clean up in that area. It has been reported that production of biosurfactants enhances the bacterial oil degradation (Matvyeyeva et al., 2014). Biosurfactants are active molecules that lower the interfacial tension between two immiscible liquids. Sarafin et al. (2014) have highlighted

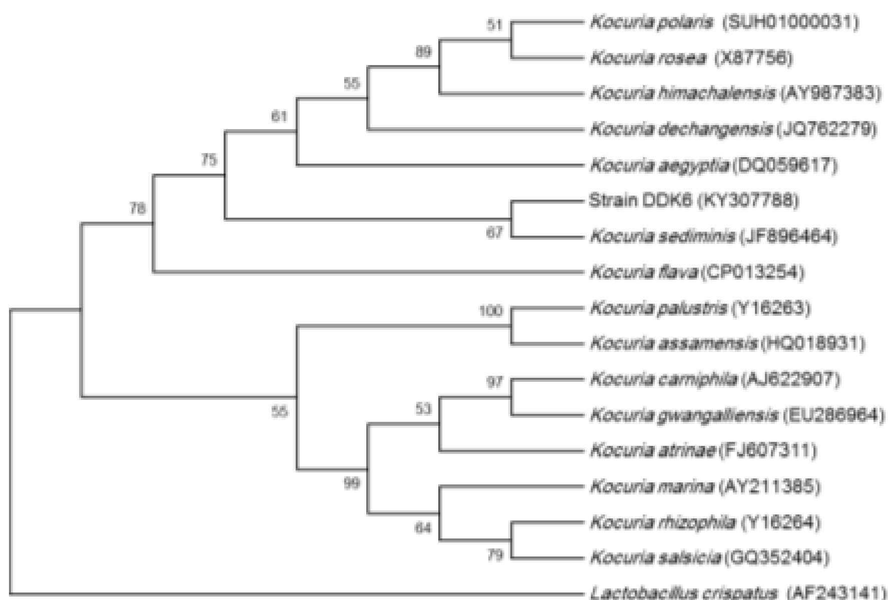

Figure 1. Neighbor-joining tree based on 16S rDNA gene sequences revealing the phylogenetic relationships between *K. sediminis* strain DDK6 accession number KY307788 and other closely related bacterial species. Accession number is given between parentheses after each bacterial species. The percentage numbers above each branch indicate the 567 levels of bootstrap support (>50%) for the branch point based on 1,500 568 resamplings. The bar represents 0.02 substitutions per site.

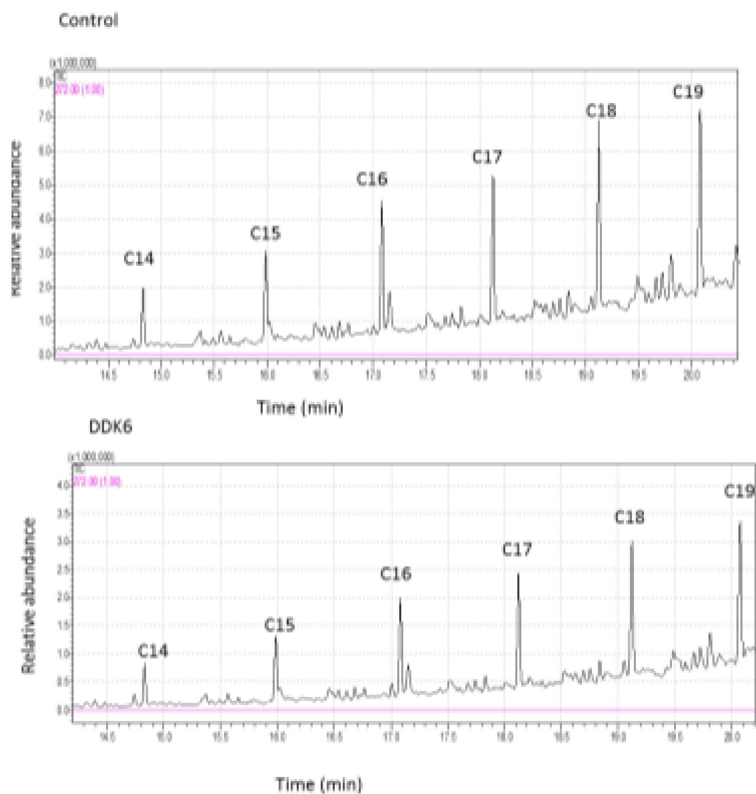

Figure 2. The GC-MS chromatogram of diesel oil extracted from culture; control (top graph) uninoculated culture and DDK6 culture (Bottom graph).

Table 3. A list of *Kocuria* spp. that possess the cytochrome P450.

Number	Name	Accession number
1	*K. rhizophila*	WP_012399225.1
2	*K. salsicia*	WP_055082043.1
3	*K. rhizophila*	WP_059281539.1
4	*K. rhizophila*	WP_019310814.1
5	*K. rhizophila*	WP_039100753.1
6	*K. rhizophila*	WP_047979332.1
7	*K.* sp. HMSC066H03	WP_070637452.1
8	*K. varians*	WP_068469792.1
9	*K. rhizophila*	WP_058954974.1
10	*K. marina*	WP_035965726.1
11	*K.* sp. ICS0012	WP_064845916.1
12	*K. flava*	WP_058857146.1
13	*K.* sp. UCD-OTCP	WP_017833470.1
14	*K. polaris*	WP_035928135.1
15	*K. polaris*	WP_058873945.1
16	*K.* sp. SM24M-10	WP_047802943.1
17	*K. turfanensis*	WP_062735211.1

that *K. marina* BS-15 produced biosurfactants reflecting its efficiency in oil degradation via increasing its water solubility to facilitate enzyme attack.

Generally, microbial degradation of diesel oil and other hydrocarbon is attributed to cytochrome P450, haem-thiolate monooxygenases, which catalyze the oxidative addition of atomic oxygen to the C-H or C-C bond of the organic compound (Van Beilen and Funhoff, 2007). Data mining for the Kocurial species that possess Cytochrome P450 showed that 17 different putative Cytochrome P450 proteins were found in 11 species (Table 3). Testing these species is crucial to confirm the ability to degrade diesel. Other enzymes such as soluble and particulate methane monoxygenases are involved in hydrocarbon degradation (Das and Chandran, 2011).

In conclusion, the cultural, biochemical and molecular characteristics were in general agreement for the strain DDK6 identification as *K. sedimins*. DDK6 exhibited a metabolic versatility and ability to degrade diesel oil indicating ecological and environmental merits for its application in bioremediation of hydrocarbon pollutants. Nonetheless, further studies are required to investigate the efficiency of the strain DDK6 for biodegradation of different organic pollutants and enzymes involved in this process at the molecular level, to explore the potentialities of the strain for biotechnological exploiting. Bearing in mind the efficiency of local bacterial strains in hydrocarbon-degradability was found to be substantially higher than that of the introduced strains (Wu et al., 2013), this could be attributed to outcompeting, biotic and/or abiotic interacting factors. This is the first report addresing *K. sediminis* as an oil-degrading bacterium isolated from an oil-contaminated soil exposed to the prevailing conditions in Al-Hofuf, Saudi Arabia.

CONFLICT OF INTERESTS

The authors has not declared any conflict of interests.

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REFERENCES

Abouseada N, Moheb M, Moez P (2016). Impact of MALDI-TOF in the Routine Diagnostic Microbiology Laboratory in Alexandria University, Egypt. Egypt. J. Med. Microbiol. 3:616-620.

Achal V, Pan X, Zhang D (2011). Remediation of copper-contaminated soil by *Kocuria flava* CR1, based on microbially induced calcite precipitation. Ecol. Eng.10:1601-1605.

Arulazhagan P, Al-Shekri K, Huda Q, Godon JJ, Basahi JM, Jeyakumar D (2017). Biodegradation of polycyclic aromatic hydrocarbons by an acidophilic *Stenotrophomonas maltophilia* strain AJH1 isolated from a mineral mining site in Saudi Arabia. Extremophiles, 1:163-174.

Austin RN, Groves JT (2011). Alkane-oxidizing metalloenzymes in the carbon cycle. Metallomics, 8:775-87.

Bala M, Kaur C, Kaur I, Khan F, Mayilraj S (2012). *Kocuria sediminis* sp. nov, isolated from a marine sediment sample. Antonie Van Leeuwenhoek. 3:469-478.

Castro DB, Pereira LB, e Silva MVM, da Silva BP, Palermo BRZ, Carlos C., ... and Gomes MB (2015). High-quality draft genome sequence of *Kocuria marina* SO9-6, an actinobacterium isolated from a copper mine. Genomics data, 5:34-35.

Chatzigeorgiou KS, Sergentanis TN, Tsiodras S, Hamodrakas SJ, Bagos PG (2011). Phoenix 100 versus Vitek 2 in the identification of gram-positive and gram-negative bacteria: A comprehensive meta-analysis. J. Clin. Microbiol. 49:3284-3291.

Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW (2007). Extaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int. J. Syst. Evol. Microbiol. 57(10):2259-2261.

Ciric L, Philip JC, Whiteley AS (2010). Hydrocarbon utilization within a diesel-degrading bacterial consortium. FEMS Microbiol. Lett. 303:116-122.

Das N, Chandran, P (2010). Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol. Res. Int. 2011:1-13

Esmaeil AS, Drobiova H, Obuekwe C (2009). Predominant culturable crude oil-degrading bacteria in the coast of Kuwait. Int. Biodeter. Biodegr. 4:400-406.

Egamberdieva D (2008). Plant growth promoting properties of rhizobacteria isolated from wheat and pea grown in loamy sand soil. Turk. J. Biol. 1: 9-15.

Hamada M, Shibata C, Tamura T, Nurkanto A, Ratnakomala S, Lisdiyanti P, and Suzuki KI (2016). *Kocuria pelophila* sp. nov, an actinobacterium isolated from the rhizosphere of a mangrove. Int. J. Syst. Evol. Microbiol. 9:3276-3280.

Hassan RM, Bassiouny DM, Matar Y (2016). Bacteremia Caused by *Kocuria kristinae* from Egypt: Are There More? A Case Report and Review of the Literature. Case Reports in Infectious Diseases. 31:1-4.

Hou D, Shi Z, Shen X, He Y, Sun M, Wang Q (2013). Isolation, identification and alkane hydroxylase genes detection of a marine diesel-degrading bacterial strain (F9). Afr. J. Microbiol. Res. 22:2794-2802.

Jirasripongpun K (2002). The characterization of oil-degrading microorganisms from lubricating oil contaminated (scale) soil. Lett. Appl. Microbiol. 35:296-300.

Khalifa AY, Almalki MA (2015). Isolation and characterization of an endophytic bacterium, *Bacillus megaterium* BMN1, associated with root-nodules of *Medicago sativa* L. growing in Al-Ahsaa region, Saudi Arabia. Ann. Microbiol. 2:1017-1026.

Kovács G, Burghardt J, Pradella S, Schumann P, Stackebrandt E, Màrialigeti K (1999). *Kocuria palustris* sp. nov. and *Kocuria rhizophila* sp. nov, isolated from rhizoplane of the narrow-leaved cattail (*Typha angustifolia*). Int. J. Syst. Bacteriol. 49:167-173.

Mariano AP, Kataoka AG, Angelis DF, BonottoDM (2007). Laboratory study on the bioremediation of diesel oil contaminated soil from a petrolstation, Braz. J. Microbiol. 2:346-352.

Matvyeyeva OL, Vasylchenko OA, Aliieva OR (2014). Microbial biosurfactant role in oil products biodegradation. Int. J. Environ. Biorem. Biodegrade. 2:69-74.

Oyehan, TA, Al-Thukair AA (2017). Isolation and characterization of PAH-degrading bacteria from the Eastern Province, Saudi Arabia. Mar. Pollut. Bull. 2:39-46.

Paim TGDS, Cantarelli VV, d'Azevedo PA (2014). Performance of the Vitek 2 system software version 5.03 in the bacterial identification and antimicrobial susceptibility test: evaluation study of clinical and reference strains of Gram-positive cocci. Rev. Soc. Bras. Med. Trop. 3:377-81.

Palomo S, González I, de la Cruz M, Martín J, Tormo JR, Anderson M, Hill R, Vicente F, Reyes F, Genilloud O (2013). Sponge-Derived *Kocuria* and *Micrococcus* spp. as sources of the new thiazolyl peptide antibiotic Kocurin. Mar. Drugs. 4:1071-1086.

Ramasamy S, Arumugam A, Chandran P (2017). Optimization of *Enterobacter cloacae* (KU923381) for diesel oil degradation using response surface methodology (RSM). J. Microbiol. 2:104-111.

Safdari MS, Kariminia HR, Nejad ZG, Fletcher TH (2017). Study Potential of Indigenous *Pseudomonas aeruginosa* and *Bacillus subtilis* in Bioremediation of Diesel-Contaminated Water. Water Air Soil Pollution 1:37-41.

Sarafin Y, Donio MBS, Velmurugan S, Michaelbabu M, Citarasu T (2014). Kocuria marina BS-15 a biosurfactant producing halophilic bacteria isolated from solar salt works in India. Saudi J. Biol. Sci. 6:511-519.

Seo YB, Kim DE, Kim GD, Kim HW, Nam SW, Kim YT, Lee JH (2009). *Kocuria gwangalliensis* sp. nov, an actinobacterium isolated from seawater. Int. J. Syst. Evol. Microbiol. 59:2769-2772.

Sharifuzzaman SM, Austin B (2010). *Kocuria* SM1 controls vibriosis in rainbow trout (*Oncorhynchus mykiss*, Walbaum). J. App. microbial. 6:2162-2170.

Sharifuzzaman SM, Al-Harbi AH, Austin B (2014). Characteristics of growth, digestive system functionality, and stress factors of rainbow trout fed probiotics *Kocuria* SM1 and *Rhodococcus* SM2. Aquaculture, 418:55-61.

Stackebrandt E, Koch C, Gvozdiak O, Schumann P (1995). Taxonomic dissection of the genus *Micrococcus*: *Kocuria* gen. nov, *Nesterenkonia* gen. nov, *Kytococcus* gen. nov, *Dermacoccus* gen. nov, and *Micrococcus* Cohn 1872 gen. emend. Int. J. Syst. Bacteriol. 45:682-692.

Tumaikina YA, Turkovskaya OV, Ignatov VV (2008). Degradation of hydrocarbons and their derivatives by a microbial association on the base of Canadian pondweed. Appl. Biochem. Microbiol. 45:382-388

Tamura K, Nei M (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10:512-526.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5, Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods Mol. Biol. Evol. 28:2731-2739.

Tan-Guan-Sheng Adrian PW, Tan JWC, Wai-Fong Yin KGC (2016). Draft genome sequence of *Kocuria rhizophila* strain TPW45, an actinobacterium isolated from freshwater. J. Genomics, 4:16-18.

Van Beilen JB, Funhoff EG (2007). Alkane hydroxylases involved in microbial alkane degradation. Appl. Microbiol. Biotechnol. 1:13-21.

Wang K, Zhang L, Liu Y, Pan Y, Meng L, Xu T, Zhang C, Liu H, Hong S, Huang H, Jiang J (2015). *Kocuria dechangensis* sp. nov, an actinobacterium isolated from saline and alkaline soils. Int. J. Syst. Evol. Microbiol. 65:3024-3030.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 17:697-703.

Wu M, Chen L, Tian Y, Ding Y, Dick WA (2013). Degradation of polycyclic aromatic hydrocarbons by microbial consortia enriched from three soils using two different culture media. Environ. Pollut. 178:152-158.

Yun JH, Roh SW, Jung MJ, Kim MS, Park EJ, Shin KS, Nam YD, Bae JW (2011). *Kocuria salsicia* sp. nov, isolated from salt-fermented seafood. Int. J. Syst. Evol. Microbiol. 61:86-289.

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Full Length Research Paper

Probiotic profiling of *Leuconostoc* species isolated from a traditional fermented cassava product

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The various properties of lactic acid bacteria have made them good auxiliary in the manufacturing process in agro food industries and farms. They are widely used as probiotics which can be defined as living microorganisms that have beneficial effects on human health probiotics could replace antibiotic growth promoters in livestock without creating new threats such as that observed with antibiotics. Before their use as probiotics lactic acid bacteria require a perfect knowledge in view to their biochemical and genetic characteristics because it is difficult to differentiate morphologically some *Leuconostoc* and *Lactobacillus* strains using morphological characteristics. This study was undertaken in order to evaluate the probiotics potential of *Leuconostoc* strains isolated from traditional fermented cassava. The results showed that 5 strains of *Leuconostoc* have antibacterial activity against *Staphylococcus aureus* (MetiR), *Klebsiella pneumoniae* (BLSE), *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*. The molecular identification of species using the conserved region of the 16S rRNA helped distinguish the species *Leuconostoc mesenteroides*. All these results showed that the studied *Leuconostoc* strains could be used as potential probiotics for the biopreservation of various foods.

Key words: *Leuconostoc*, antibacterial activity, polymerase chain reaction (PCR), sequencing, probiotics.

INTRODUCTION

Probiotics are defined as living microorganisms that have beneficial effects on human health (FAO/WHO, 2002). Indeed, several studies have demonstrated that probiotics may enhance growth performance, immunity and disease resistance (Saxelin et al., 2005; Ezendam and van Loveren, 2006; Ström-Bestor and Wiklund, 2011). Despite the numerous definitions, the criteria to select probiotic strains are total safety for the host, resistance to gastric acidity and pancreatic secretions, adhesion to epithelial cells, antimicrobial activity, inhibition of adhesion of pathogenic bacteria, evaluation of resistance to antibiotics, tolerance to food additives and stability in the food matrix (Soccol et al., 2010). In addition, the functional properties of probiotics include

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hypocholesterolemic activity by lowering plasma cholesterol, preventing and treating diarrhoea (Liong and Shah, 2005). The mechanisms by which probiotics exert their beneficial effects on the host include the reduction of luminal pH, competition with pathogens for adhesion sites and nutritional sources, secretion of antimicrobial substances, toxin inactivation, and immune stimulation (Salminen et al., 2004). The most commonly used probiotics are the strains of lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus* (*Streptococcus thermophilus*); the first two are known to resist gastric acid, bile salts and pancreatic enzymes, to adhere to colonic mucosa and readily colonize the intestinal tract (Fioramonti et al., 2003).

The use of selected probiotics from alternative sources known as “unconventional sources” is likely to increase. These unconventional sources include non-intestinal sources and non-dairy fermented food products, such as traditional fermented foods, traditional fermented drinks, vegetables, and fruit juices (Ramirez-Chavarin et al., 2013; Siddiqee et al., 2013). In Côte d’Ivoire, cassava (*Manihot esculenta*) root is traditionally fermented into a traditional microbial starter called “mangnan” that is used to prepare a really appreciated food called “attiéké” defined as fermented and steamed semolina cassava (Assanvo et al., 2006; Dje et al., 2008). Studies on this traditional microbial starter showed that the dominant microflora consists of lactic acid bacteria (LAB) (Assanvo et al., 2006). LAB, a group of Gram-positive, non-spore forming, non-motile microorganisms can produce inhibitory compounds such as lactic acid, bacteriocin and hydrogen peroxide preventing the growth of harmful microorganisms. The role of LAB in improving the shelf life and nutritional quality of fermented foods and beverages, controlling diarrhea, as well as their antimicrobial properties have also been established. However, despite an increasing interest in LAB, there is a paucity of literature regarding novel and emerging uses of LAB as probiotics, especially from traditional African fermented foods. Thus, the objective of the current study was to characterize the potential probiotic properties of *Leuconostoc* species isolated in previous studies (Coulibaly et al., 2016) from traditional fermented cassava.

MATERIALS AND METHODS

LAB and indicator strains

The microbial strains used in this study are shown in Table 1. Five (05) LAB belonging to *Leuconostoc* genus, previously isolated from traditional fermented cassava (Coulibaly et al., 2016) were used as test strains: BL1, BL2, BL7, BL39, BL44, and BL61. The LAB reference strains used were *Lactobacillus plantarum* CWBIBF-76, *Enterococcus faecium* EFTHT and *Weissella confusa* CWBI-B902. The indicators strains (Table 1) were collected from Institute Pasteur of Côte d’Ivoire.

Table 1. LAB and indicators strains.

Test strains
BL1
BL7
BL39
BL44
BL61
Lactobacillus plantarum CWBI BF-76
Enterococcus faecium EFTHT
Weissella confusa CWBI-B902
Indicator strains
Staphylococcus aureus
Escherichia coli
Salmonella typhimurium
Stqphylococcus hemolyticus (Resistant to methicillin: SARM)
*Klebsiella pneumoniae (*Producer of beta-lactamase: ESBL)
Staphylococcus aureus ATCC 25923
Escherichia coli ATCC 25922
Pseudomonas aeruginosa ATCC 27853

Antimicrobial activity determination

The method of agar spots as described by Larpent-Gourgaud et al. (1997) was used to evaluate the antimicrobial activity of the selected LAB strains. For this Petri dish, MRS agar was spotted with a 24-h colony of the LAB strain. The plates were seeded at 37°C for 24 h. At the same time, the indicator strains were subcultured in BCC broth for 3 h and then isolated on selective agar and incubated at their optimum growth temperature for 18 h. Each indicator strain was suspended in 2 ml of 0.85% NaCl and then vortexed. The OD was adjusted to 2.5 Mc Farland. Then, the inoculum was obtained by mixing 1 ml of inoculum of each strain in 9 ml of physiological water. The boxes with the spots were inoculated by flooding and the dishes were observed after 24 h of incubation at 37°C. The size of the zones of inhibition produced was measured.

Morphological, physiological and biochemical identification

The Gram characteristics of the isolates were determined using light microscope (Leica DM 1000, France) following staining. LAB are known to be Gram-positive. Cultures were grown in appropriate MRS media at 37°C for 48 h under anaerobic conditions. Cells from fresh cultures were used for Gram staining.

The determination of fermentation profiles (heterofermentative or homofermentative) was performed by inoculating 10 ml MRS broth containing bell Durhams at 28°C for 24 h (Harir et al., 2009).

For the fermentation of sugars, MRS media broth without glucose and supplemented with bromocresol purple as indicator was used (Mannu et al., 2000). For this, 9 ml of the medium is left in test tubes and sterilized for15 min. One milliliter of the sugar solution (10%, p/v) was aseptically added after filtration on a membrane with 0.45 µm porosity. Incubation was performed at 28°C for 48 h. Tested sugars were arabinose, sucrose, fructose, trehalose and esculin (Garvie, 1983).

The method described by Leveau et al. (1991) was used to determine the dihydrolase arginine (ADH), while the ability to grow at 10, 37 and 44°C was determined after incubation of inoculated Petri dishes at these temperatures.

Table 2. Features of primers used for PCR.

Primers	Tm (°C)	%GC	Sequences (5'--- 3')	Reference
16F27	57.3	50	AGAGTTTGATCCTGGCTCAG	Bayane et al. (2006)
16R1522	44.7	60	AAGGAGGTGATCCAGCCGCA	

Tm: Hybridization temperature; % GC: guanine and cytosine percentage.

Table 3. Diameter of inhibition (mm) of indicators strains.

Test strains	Indicators strains						
	S. aureus	*E. coli*	*S.* *Typhimurium*	*S. hemolyticus* MétiR	*K. pneumoniae* (BLSE)	*S. aureus* ATCC 25923	*E. coli* ATCC 25922
BL1	19	12	20	19	17	22	15
BL7	17	11	14	16	16	20	18
BL39	19	16	17	24	21	24	20
BL44	22	13	18	30	19	26	22
BL61	20	11	20	31	19	17	22
Lactobacillus plantarum CWBI BF-76	20	19	22	22	20	23	16
Enterococcus faecium EFTHT	10	8	11	11	11	23	21
Weissella confusa CWBI-B902	15	8	10	16	8	9	12

Molecular identification

DNA extraction and 16s rDNA amplification

A colony from culture was resuspended in 300 µl pure water in 1.5 ml Eppendorf tubes. DNA isolation and purification were realized using *Instagen Matrix kit* (Bio-Rad, USA) according to the manufacturer's instructions. PCR targeting the 16s rDNA of LAB were done as described by Bayane et al. (2006). The amplification reactions of 16S rDNA region were realized in a final volume of 50 µl containing 1X Master Mix (5 PRIME HotMasterMix, 5PRIME), 1 µl of DNA template (approximately 50 ng), 1 µM of each primer (Table 2) and PCR grade water.

The amplification was carried out in a 2720 ABI thermalcycler (Applied Biosystems, Syngapore). PCR conditions started with an initial denaturation at 94°C for 2 min; 36 cycles consisting of 1 min denaturation at 94°C for 30 s, annealing at 58°C and elongation at 65°C for 2 min. Then, a final extension for 7 min at 65°C ended the PCR reaction. PCR products were revealed in 1% agarose gel electrophoresis containing ethidium bromide (1 µg/ml).

Sequencing of the amplified DNA and data analysis

PCR products were sent to a Company (GATC BIOTECH, GERMANY) for sequencing. Sequences obtained were compared to those listed in Genbank (National Center for Biotechnology Information) using the nucleotid BLAST 2.5.0 tool (Zheng et al., 2000; Aleksandr et al., 2008). Similarity percentages were determined between the isolated sequences in this study and the closest sequences listed in GenBank. Sequences were considered similar when they have at least 99% percentage of similarity. Phylogenetic constructions were done after re-alignment of the sequences using MEGA 7.0.14 (Kumar et al., 2016). The maximum likelihood and UPGMA algorithms (USA) were chosen for trees construction.

RESULTS AND DISCUSSION

Antibacterial activity

Among the nine LAB strains tested for antibacterial activity in solid medium, only 5 strains (BL1, BL7, BL39, BL44, and BL61) showed good activity against all the indicator strains. The inhibition diameters measured are superior or equals to those of reference strains (*L. plantarum*, *E. faecium*, and *W. confusa*) (Table 3).

Inhibition diameter is denoted positive when greater than 8 mm (Schillinger et al., 2001). BL39, BL44 and BL61 strains inhibited the growth of all indicators Gram positive strains *Staphylococcus hemolyticus* MetiR and *Staphylococcus aureus* ATCC 25923.

Bacteria of the genus *Leuconostoc,* in association with the mesophilic LAB are capable of inhibiting the growth of pathogenic microorganisms such as *S. aureus*, *Klebsiella pneumoniae* BLSE, *Escherichia coli, Salmonella* Typhimurium and reference test strains, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Indeed, Todorov and Dicks (2005) have shown the growth inhibitory of *E. coli* and *P. aerugionosa* by a product of *Leuconostoc mesenteroides* subsp. *mesenteroides*. The antagonistic action of LAB against pathogens such as *Salmonella*, *S. aureus,* and *E. coli* was also confirmed by studies of Makras et al. (2006).

The diameters of inhibition of the indicator strains *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were higher than those of the three reference strains. The strain BL44 showed a strong

Table 4. Biochemical characteristics of the selected *Leuconostoc* strains.

Parameter	Strains				
	BL1	**BL7**	**BL39**	**BL44**	**BL61**
Arabinose	-	+	+	+	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Trehalose	+	+	+	+	+
Hydrolysis of esculin	-	+	+	-	+
ADH	-	-	-	-	-
Type of fermentation	He	He	He	He	He
10°C / 72 h	+	+	+	+	+
37°C 24 h	+	+	+	+	+
40°C / 24 h	+	+	+	+	+
Morphology	Cocci Gram+	Cocci Gram+	Cocci Gram+	Cocci Gram+	Cocci Gram+

He: Heterofermentative.

inhibition (60 mm) of growth of the *P. aeruginosa* ATCC 27853. All strains diameters inhibition of *E. coli* and *Staphylococcus* were similar to those obtained by Labiou et al. (2005).

The antagonist effect as shown is this study is related to the biosynthesis of inhibitor compounds observed in strains of lactic bacteria (Servin, 2004). Indeed, organic acids are able to acidify the cytoplasm after dissociation and inhibit the cellular enzymatic activity of acid-sensitive pathogens (Tou et al., 2006; Djéni et al., 2008). This decrease in pH can therefore affect the viability of pathogenic bacteria (Bruno et al., 2002; Servin, 2004). This inhibition effect may be related to a competition with nutrients. An increase in the number of LAB obtained during a probiotic treatment would make it possible to reduce the substrates available for the implantation of pathogenic microorganisms (Fooks and Gibson, 2002). This justify the fact that all bacteriocins produced by LAB antimicrobial activity against Gram + (Dortu and Philippe, 2009).

Leuconostoc producing bacteriocin can be found in different food products including meat, cereals and milk (Hastings et al., 1994; Wulijideligen et al., 2012).

Morphological, biochemical and physiological characteristics

Morphological, biochemical and physiological examinations of the strains with antibacterial activity are shown in Table 4. Bacterial cells were spherical grouped into short chains and diplococcic. All strains were heterofermentative after 24 h. None of strains possessed hydrolase arginine.

Phylogenetic analysis and identification

The PCR targeting the 16 rDNA showed that all the strains that have demonstrated antibacterial activity belong to the lactic bacteria family. Positive samples detected highlighted a 1500 bp PCR product on agarose gel (Figure 1).

Sequences analysis and phylogeny

The 5 *Leuconostoc* PCR 16 S rDNA products were sequenced. The BLAST*n*'s results confirmed that all the 5 isolates BL1, BL7, BL39, BL44 and BL 61 were genetically related to *L. mesenteroides* with 99% identity. The multiple sequence alignment of the Ivoirian *Leuconostoc* strains with other isolated strains from other region of the world show that all the 5 Ivoirian strains presented identical sequence. The genetic tree analysis showed clearly that those strains form a new genetic group related to *L. mesenteroides* on the basis of Blast results but are genetically distant (Figure 2). The 16S rRNA gene sequences of BL1, BL7, Bl39, BL44, and BL61 where deposited in GenBank nucleic acid sequence database under accession number KM518656 to KM518660.

At genetically sequence alignment of 16S rRNA of BL1, BL7, BL39, BL44 and BL61 strains showed that they were close to *L. mesenteroides* ssp. *mesenteroides* and *L. mesenteroides* ssp. *dextranicum.*

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Zheng et al., 2000). The tree with the highest log likelihood (-2249.2146) is as shown in Figure 2. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions

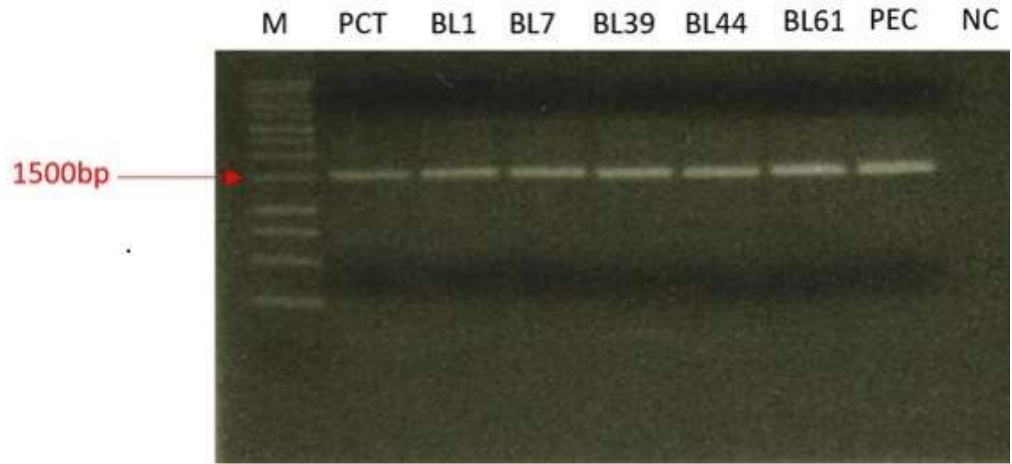

Figure 1. Agarose gel obtained after electrophoresis of PCR's16sDNA product from *Leuconostoc* strains. M: Molecular weight marker (1 kb DNA Ladder); PCT: positive PCR test control; NC: negative control; *Leuconostoc* strains: BL1, BL7, BL39, BL44, BL61.

Figure 2. Molecular phylogenetic analysis by Maximum Likelihood method of *Leuconostoc* strains isolated from traditional fermented Cassava.

per site (next to the branches). The analysis involved 22 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 515 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Aleksandr et al., 2008).

Conclusion

In conclusion, it can be stated that strains of LAB that have antibacterial properties belong to *L. mesenteroides*. In view to their strong inhibitory properties on *P. aeruginosa* and on methicillin-resistant *S. aureus*, these strains could be used as biopreservatives of various foods. Furthermore, a better knowledge of other criteria for selecting the probiotics associated with technological properties would make them potential candidates for the formulation of ready to use probiotics

CONFLICT OF INTERESTS

The author(s) have not declared any conflict of interest.

REFERENCES

Aleksandr M, George C, Yan R, Thomas LM, Richa A, Alejandro AS (2008). Database Indexing for Production MegaBLAST Searches Bioinformatics 24:1757-1764.

Assanvo JB, Agbo GN, Behi YEN, Coulin P, Farah Z (2006). Microflora of traditional starter made from cassava for attiéké production in Dabou (Côte d'Ivoire). Food Control 17:37-41.

Bayane A, Dominique R, Robin DD, Jacqueline D, Brehima D, Philippe T (2006). Assessment of the physiological and biochemical characterization of a *Lactic acid bacterium* isolated from chicken faeces in sahelian region. Afr. J. Biotechnol. 5(8):629-634.

Bruno FA, Lankaputhra WEV, Shab NP (2002). Growth, viabilty and activity of bifidobacterium spp in skim milk containing prebiotics. J. Food Sci. 67:2740-2744

Coulibaly KE, Coulibaly KJ, Goualié GB, Akpa EE, Niamké SLA, Mireille D (2016). Benchmarking Loads of Lactic Acid Bacteria from Traditional Ferment of Cassava Used for the Preparation of Cassava Meal in Abidjan. Int. J. Curr. Res. Biosci. Plant Biol. 3(8):69-73.

Djeni NT, N'guessan KF, Dadie AT, Dje KM (2008). Impact of different rates of a traditional starter on biochemical and microbiological changes during the fermentation of cassava for attiéké production. Food 2:145-151.

Dortu C, Philippe T (2009). Les bactériocines des bactéries lactiques: caractéristiques et intérêts pour la bio conservation des produits alimentaires. Biotechnol. Agron. Soc. Environ. 13(1):143-154.

Ezendam J, van Loveren H (2006). Probiotics: immunomodulation and evaluation of safety and efficacy. Nutr. Rev. 64:1-14

FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. Report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food. World Health Organization, London Ontario, Canada, 2002: 8. Available from: ftp://ftp.fao.org/es/esn/food/wgreport2.pdf

Fioramonti J, Theodorou V, Bueno L (2003). Probiotics: What are they? What are their effects on gut physiology?, Best Pract. Res. Clin. Gastroenterol. 17:711-724.

Fooks IJ, Gibson GR (2002). Probiotics as modulators of the gut flora. Br. J. Nutr. 88(1):S39-S49

Garvie EI (1983). *Leuconostoc mesenteroides subsp cremoris* (Knudsen and Sorensen) comb. Nov. and *Leuconostoc mesenteroides subsp dextranicum* (Beijerinck) comb. nov. Int.J. Syst. Bacterial. 33: 118-119.

Harir A, Ouis N, Sahnouni F, Bouhadi D (2009). Mise en œuvre de la fermentation de certains ferments lactiques dans des milieux à base d'extraits de carouche. Rev. Microbiol. Ind. San. Environ. pp. 37-55.

Hastings JW, Stiles ME, Von HA (1994). Bacteriocins of leuconostocs isolated from meat Int. J. Food Microbiol. 24(1-2):75-81.

Kumar S, Stecher G, Koichiro T (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33(7):1870-1874.

Labiou H, Elmoualdi L, El Yachioui M, Ouhssine M (2005). Sélection de souches de bactéries lactiques antibactériennes. Bull. Soc. Pharm. Bordeaux 144:237-250.

Larpent-Gourgaud M, Odile M, Larpent J-P, Desmasures N, Desmazeaud M, Mangin I, Masson F, Montel M-C, Patrick T (1997). Les ferments lactiques et bactéries apparentées. In. Microbiologie alimentaire techniques de laboratoire. Pp.199-255.

Leveau J-Y, Marielle Bouix, H de Roissart (1991). La flore lactique. In. Techniques d'analyses et de contrôle dans les industries agroalimentaires : le contrôle microbiologique, C. M. Bourgeois et Leveau, J.-Y. Pp. 152-186

Liong MT, Shah P (2005). Optimization of cholesterol removal by probiotics in the presence of prebiotics by using a response surface method. Appl. Environ. Microbiol. 71(4):1745-1753.

Makras L, Triantafyllou V, Fayol-Messaoudi D, Adriany T, Zoumpopoulou G, Tsakalidou E, Servin A, De Vuyst L (2006). Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards Salmonella enterica serovar Typhimurium reveals a role for lactic acid and other inhibitory *compounds*. Res. Microbiol. 157(3):241-247.

Mannu L, Paba A, Pes M, Scintu MF (2000). Genotypic and phenotypic heterogeneity among lactococci from traditional Pecorino Sardo Cheese. J. Appl. Microbiol. 89:191-197.

Ramirez-Chavarin ML, Wacher C, Eslava-Campos CA, Perez-Chabela ML. (2013). Probiotic potential of thermotolerant lactic acid bacteria strains isolated from cooked meat products. Int. Food Res. J. 20:991-1000.

Salminen S, Wright AV, Ouwehand A (2004). Lactic acid bacteria. microbiological and functional aspects. Marcel Dekker. Inc., U.S.A.

Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM (2005). Probiotic and other functional microbes: from markets to mechanisms. Curr. Opin. Biotechnol. 16:204-211.

Schillinger U, Becker B, Vignolo G, Holzapfel WH (2001). Efficacy of nisin in combinaison with protective culture against Listeria monocytogenes Scott A in tofu. Int. J. Food Microbiol. 71:159-168

Servin A (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. 28:405-440

Siddiqee MH, Sarker H, Shurovi KM (2013). Assessment of probiotic application of lactic acid bacteria (LAB) isolated from different food items. Stamford J. Microbiol. 2:10-14.

Soccol CR, Vandenberghe LP, Spier MR, Medeiros AB, Yamaguishi CT, Lindner JD, Pandey A, Thomaz-Soccol V (2010). The Potential of Probiotics: A Review. Food Technol. Biotechnol. 48(4):413-434.

Ström-Bestor M, Wiklund T (2011). Inhibitory activity of *Pseudomonas sp.* on Flavobacterium psychrophilum, in vitro. J. Fish Dis. 34:255-264.

Todorov SD, Dicks LMT (2005). Production of Bacteriocin ST33LD, produced by *Leuconostoc mesenteroides* subsp. *mesenteroides*, as Recorded in the Presence of Different Medium Components. World J. Microbiol. Biotechnol. 21(8):1585-90.

Tou EH, Guyot JP, Mouquet-Rivier C, Rochette I, Counil E, Traoré AS, Treche S (2006). Study through surveys and fermentation kinetics of the traditional processing of pearl millet (Pennisetum glaucum) into bensaalga, a fermented gruel from Burkina Faso. Int. J. Food Microbiol. 106:52-60.

Wulijideligen, Asahina T, Hara K, Arakawa K, Nakano H, Miyamoto T (2012). Production of bacteriocin by *Leuconostoc mesenteroides* 406 isolated from Mongolian fermented mare's milk, airag. Anim. Sci. J. 83:704-711.

Zheng Z, Scott S, Lukas W, Webb M (2000). A greedy algorithm for aligning DNA sequences J. Comput. Biol. 7(1-2):203-214.

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Full Length Research Paper

Characteristics of cellulase in cellulose-degrading bacterium strain *Clostridium straminisolvens* (CSK1)

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Microbial degradation of biomass was considered as a clean and highly efficient approach to produce bioenergy, as it could mitigate the urgent demand for limited petroleum and natural gas. A thermophilic microbial consortium (MC1) was proved a high efficiency cellulose-degrading bacterial community in previous studies. A novel anaerobic, thermophilic, and cellulolytic bacterium (strain CSK1) was isolated from MC1. The cellulase activity characteristics of CSK1 were analyzed and evaluated by exploring new measuring conditions via the 3,5-dinitrosalicylic acid (DNS) spectrophotometry and the carboxymethyl cellulose (CMC) saccharogenic power method. The results indicated that the optimal measuring wavelength, reaction temperature, and pH value, were 530 nm, 60°C and 6.0, respectively. The ideal reaction time to achieve stable and significant measuring cellulase activity was about 10 min. Cellulase of CSK1 remained stable when the temperature was below 70°C, and the pH between 5.0 and 10.0, and its activity was quickly reduced when the temperature and pH exceeded such ranges. The cellulase activity of CSK1 reached the highest level on culturing day 8, and high correlations were found among cellulase activity variation, pH and CSK1 biomass change.

Key words: Strain CSK1, cellulose degradation, composite microbial system microbial consortium (MC1), 3,5-dinitrosalicylic acid (DNS) spectrophotometry, enzyme activity.

INTRODUCTION

With the intensified crisis of global petroleum supply and climate change, the urgent demand in the production of sustainable and renewable energy becomes an important issue all over the world (Stephanopoulos, 2007; Kerr, 2007). As one of the most widely used renewable energy, bioenergy has high potential in alleviating the energy crisis. Among all the possible materials for production of bioenergy, cellulosic resources have their distinct advantages compared to others, such as widespread distribution, no competition with food production, low pollution, and sustainability (Bugg et al., 2011). However, the natural structure of cellulosic resources is a big obstacle for utilizing them to produce bioenergy and renewable chemicals: Cellulose is embedded in a lignin matrix, which has an insoluble high crystal structure framework, and is difficult to hydrolyze. Such structure has largely limited the full utilization of the available cellulosic resources.

Microorganisms provide many advantages as potential sources of cellulosic material degradation: They can

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degrade various types of materials, pose little threat to the environment, are highly efficient at degradation and are reusable. Numerous studies have been conducted on the degradation of cellulosic materials by using different types of microorganisms (Anand et al., 2010; Bayer et al., 1998; Pérez et al., 2002; Wang and Gao, 2003; Xu and Goodell, 2001). However, without pre-treatment and/or sterilization of cellulosic materials, effective utilization of these microorganisms for such causes remains difficult. Cellulosic materials are naturally degraded by the cooperation of many microorganisms working in tandem. Previous studies have shown that an ideal culture for cellulose degradation was a mixed culture comprised of a cellulolytic bacterium and a non-cellulolytic bacterium (Guo et al., 2010; Lewis et al., 1988; Lü et al., 2013; Odom and Wall, 1983).

In our past research, a stable bacterial community, MC1 (thermophilic microbial consortium), was constructed that was capable of effectively degrading various cellulosic materials (for example, Filter paper, cotton and rice straw) under aerobic static conditions at 50°C (Cui et al., 2002). It was capable of completely degrading 0.5 g/100 mL filter paper within 48 h, and of completely degrading 2 g/100 mL rice straw within 8 days (Wang et al., 2005). The cellulose-degradation efficiency and the composition of this bacterial community have remained unchanged for over 10 years. To fully understand the mechanisms responsible for the effective cellulose degradation, the characteristics of each individual bacterium must be clarified (and especially those of the cellulose-degrading bacterium). CSK1 (*Clostridium straminisolvens*) is the only cellulose-degrading bacterium that has been successfully isolated from MC1 (Kato et al., 2004a, b). Researchers have studied the relationship between CSK1 and other bacterium strains in MC1 (Kato et al., 2008), and the loss of the substrate weight of CSK1 (Kato et al., 2004b). However, no research has been done by using cellulase characteristics to evaluate the cellulase production and the cellulose degradation ability of CSK1. Cellulase is a multicomponent enzyme, for which composition and proportion can be significantly different. Also, the substrates with which that cellulase works are themselves complex. Therefore, many methodological studies have been conducted for cellulase activity determination and evaluation (Bailey et al., 1975; Ghose 1987; Goksyr and Eriksen, 1980; Singh et al., 2009), and reported that different types of cellulase require different reaction temperatures, pH, reaction times, and other specific conditions. Characteristics of MC1 cellulase activity have been studied previously (Cui et al., 2004; Zhe et al., 2003). However, no research has been done on the determination conditions, the production mechanisms, and the evaluation of the cellulase of CSK1.

In the current study, the measuring wavelength, reaction time, temperature and pH were taken into consideration for optimizing the determination parameters of cellulase in CSK1. The stability of callulase at different pH and temperature conditions were evaluated. CSK1 was cultured for 16 days; its cellulase activitiy, pH, and protein quantity throughout the growth cycle were measured to evaluate the production mechanisms and characteristics of cellulase.

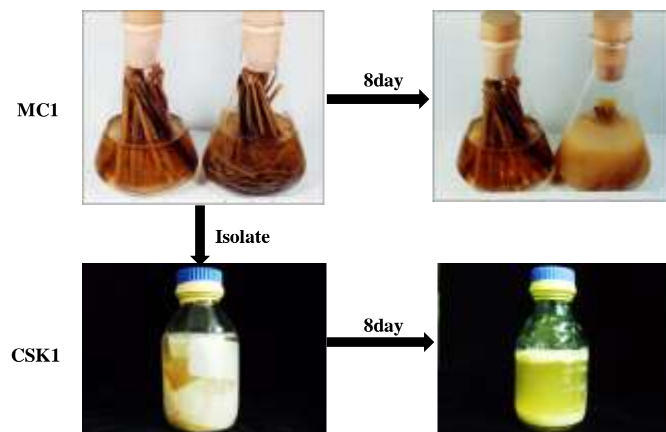

Figure 1. 2g/100mL rice straw was completely degraded within 8 days by MC1. 1g/100mL filter paper was completely degraded within 8 days by CSK1.

MATERIALS AND METHODS

Bacterial strain

CSK1 is a novel anaerobic, thermophilic and cellulolytic bacterium. The 16S rRNA gene sequence of the CSK1 stain was mapped to cluster III of the genus *Clostridium*. Strain CSK1 is closely related to *Clostridium thermocellum* (96.2%) and *Clostridium aldrichii* (95.1%) (Kato et al., 2004a). In the current research, the CSK1 strain which was anaerobically isolated from the cellulose-degrading bacterial community MC1, and stored in our lab was used for the production of cellulase for the following research (Figure 1).

Culture conditions

The inoculation and culturing were conducted under stringent anaerobic conditions. The culturing media were prepared by dissolving and thoroughly mixing 1 g yeast extract, 5 g peptone, 2 g $CaCO_3$, 5 g NaCl, 1 mg resazurin and 10 g filter paper with 1 L of water (pH 7.0). Reducing agent resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) (Sangon Biotech, Shanghai, China) was used as an oxidation-reduction indicator in culturing media. The fully reduced anaerobic culturing media, which indicated as colorless state of resazurin was used for the cultivation of CSK1 strain. The culturing media were sterilized and cooled down in an anaerobic chamber operated by an anaerobic gas mixture (H_2/N_2: 10/90, v/v). The CSK1 was inoculated and cultured in the culturing media under anaerobic conditions at 50°C for 8 days.

Preparation of crude enzyme

To estimate enzyme activity, culture samples were taken out in anaerobic condition from the previous shook cultural flask. 15 mL

centrifuge tubes, each contained 7 mL culture sample was centrifuged at 12,000 rpm/min for 10 min at 4°C. The supernatants and pellets were separated, and the supernatants were used as extracellular crude enzyme samples to determine cellulase activity.

Determination of cellulase activity

The enzyme activity of CSK1 was determined using the CMC saccharogenic power and the DNS spectrophotometry method. The CMC is water soluble, thus can be easily hydrolyzed. It was used as the substrates to determine the activity of CMCase. Basically, the CMC was degraded by the cellulase to produce free reducing sugars, which quantity was then determined by the DNS method to evaluate the cellulase activity (Aoyama et al., 2015; Eveleigh et al., 2009; Wang et al., 2014). In operation, the substrate was suspended in 1/15 mol/L disodium hydrogen phosphate-potassium-dihydrogen phosphate buffer (pH 6.24) at 1% (w/v). 0.5 mL substrate solution was pre-heated at 60°C for 5 min prior to the addition of 0.5 mL culture supernatant. The mixture, including buffer, crude enzyme solution, and substrate, was incubated for 10 min at 60°C. The DNS method was used for the measurement of enzyme activity (Ghose, 1987). All samples were analyzed in triplicate. One unit (IU) of enzyme activity was defined as the amount of enzyme releasing 1 μg of reducing sugars during a 1 min reaction.

Wavelength for cellulase activity measurement

The ultraviolet spectrophotometer (Model UV-2550, SHIMADZU, Japan) was used to scan the samples, and the measurement wavelength range from 490 to 580 nm was selected for the scanning. The absorption of DNS and its reaction products with crude cellulase extract produced by CSK1 were measured by the spectrophotometer.

Effect of reaction temperatures on cellulase activity

To determine cellulase activity at different temperatures, the cellulase reaction temperature was set as 30, 40, 50, 60, 70 and 80°C. Cellulase activity measurements at each respective temperature were conducted as described in the determination of cellulase activity section.

Effect of pH on cellulase activity

To determine cellulase activity at different pH levels, we used sodium phosphate buffer solution to modulate the reacting substrate CMC solution into different pH levels: 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0. Cellulase activity measurements were conducted as described in the determination of cellulase activity section.

Effect of reaction time on cellulase activity

To determine cellulase activity at different time periods, the reaction time was set as 5, 10, 20, 30, and 40 min. The quantity of reducing sugar produced during the reaction process, as well as the cellulase activities were measured for evaluation of the influence of the reaction time.

Effect of treatment pH on cellulase activity

Buffer solutions with different pH levels from 2 to 12 were prepared of 0.2 mol·L^{-1} sodium phosphate. 25% crude enzyme samples were added into the buffer solutions respectively, and allowed each to sit for 1 h at 30°C. Cellulase activity measurements were conducted as described in the determination of cellulase activity section. The cellulase activity of crude enzyme samples (pH 6.0) without any added sodium phosphate (possessing 100% cellulase activity) was used as the control for the overall measurement. The cellulase activities of different processed samples were compared to the control to estimate the stability of cellulase at different pH values.

Effect of treatment temperature on cellulase activity

The CSK1 crude enzyme samples were placed at constant temperatures of 30, 40, 50, 60, 70, 80, 85 and 90°C for 30 min. Then the cellulase activity of the samples was measured as described in the determination of cellulase activity section. The cellulase activity of crude enzyme samples was set as 100% cellulase activity in order to estimate the relative stability of samples processed at the above temperatures.

Characteristics of cellulase activity throughout the growth cycle

During the culturing period of CSK1, a 5 mL culture sample was extracted each day, and centrifuged at 12,000 rpm/min for 10min at 4°C to obtain the separated the supernatants and pellets. The supernatants were used to determine the cellulase activity and the protein quantity. Meanwhile, the pH of the culture solution in the growth cycle was determined and recorded each day.

RESULTS AND DISCUSSION

Optimization of measuring the wavelength for cellulase activity

There are many methods for the determination of cellulase activity, and they are diverse and complicated (Dashtban et al., 2010). DNS spectrophotometry is a widely accepted and applied method in research (Aoyama et al., 2015; Miller, 1959; Wood and Bhat, 1988; Xiao et al., 2004; Zhao et al., 2016), which determined the ability of DNS to saccharify CMC via the analysis of its hydrolysis products, such as glucose and cellobiose (reducing sugars).

The absorbance of DNS was scanned with wavelengths between 490 and 580 nm, and found that the OD values decreased from 1 to 0 with the scanning wavelengths from 490 to 530 nm (Figure 2). No absorbance was found in the DNS with scanning wavelength over 530 nm. The results indicated that the influence of DNS decreased with the increasing measuring wavelength from 490 to 530 nm. The scanning results of the reaction products from the crude cellulase extract and the chromogenic reagent DNS showed thatthe OD values increased from 490 to 500 nm, and reached the peak at 500 nm, then slowly decreased with the increasing scanning wavelength from 500 to 580 nm. Compared to the decreased rate in OD values of the DNS, the changing rates of reducing sugar were much lower, and no significant differences were found among

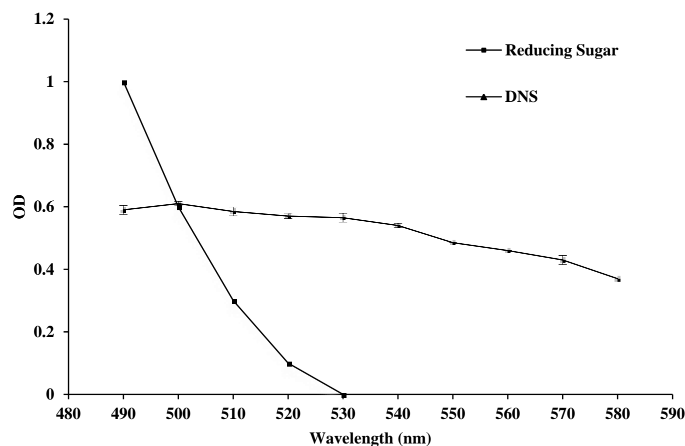

Figure 2. The absorption of DNS and reducing sugar at different wavelengths.

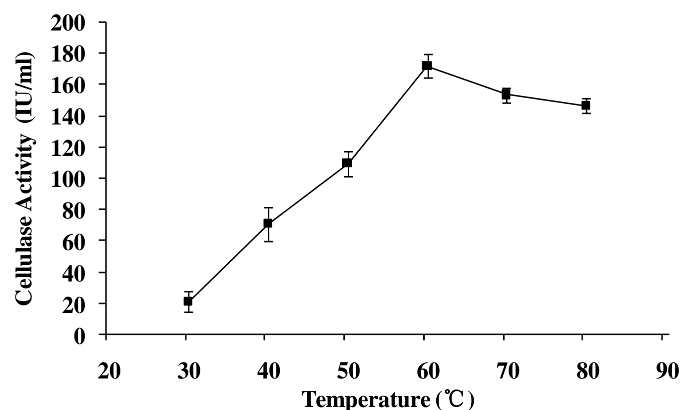

Figure 3. Cellulase activity at different reaction temperatures.

scanning wavelengths from 490 to 530 nm. Therefore, any wavelength of 490 to 530 nm was good for the measurement of OD values in the reaction products of cellulase and reagent DNS. In order to eliminate the influence of DNS and obtain more repeatable results, the measuring wavelength at 530 nm was selected for the determination of cellulose activity in CSK1.

A wide range of wavelengths has been used to determine the reducing sugars using the broadly defined DNS method (Stellmach, 1992; Zhang et al., 2009). Different measurement wavelengths resulted in different OD values, and the OD value was used to evaluate the cellulase activity, thus, as OD values shift, so will cellulase activity results.

In the current study, the absorption peak of the reducing sugar was under wavelength at 500 nm, but DNS showed obvious absorption in the 450 to 530 nm wavelength range and had an amplitude change of absorbency with an apparent declining tendency. Thus, the reducing sugar measuring results with a wavelength of 500 nm was not stable due to the influence of the high absorption values of the DNS.

In a previous study, the optimal wavelength for the cellulase activity measurement of bacterial community MC1 is at 490 nm (Piao et al., 2002); however, researchers did not take the scanning interference of DNS into consideration. Although the absorption of crude cellulase reacting to DNS of CSK1 under 530 nm declined in comparison to 500 nm, DNS interference of DNS was relatively lower. So we chose 530 nm as the optimal measurement wavelength.

Optimal temperature and pH of the cellulase reaction

Cellulase degradation has a wide application value in industrial fields concerning food, feed, medicine, textiles, detergent, and papermaking. However, it is required to fulfill special conditions in terms of pH, temperature and stability to achieve targeted activities. The growing conditions of the microorganisms are various depending on the process configurations. Thus, to study the characteristics of cellulase, especially the optimal reaction pH, temperature and its stability are critical to further understand the degradation mechanism of cellulases.

The measured cellulase activity of CSK1 increased quickly with the temperature increased from 30 to 60°C, and then gradually decreased with the increased temperature up to 80°C (Figure 3). Significant differences were found between the temperatures 60°C and 50 and 70°C, which were the two temperatures that have closer cellulase activity to 60°C. Thus, the measuring temperature at 60°C was selected for the future determination of cellulase activity. The cellulase activity of CSK1 maintained a high and consistent value around 150 IU/mL under pH 4.0 to 7.0, and reached the highest at pH 6.0 (Figure 4). The cellulase activity decreased with the increasing pH from 6 to 11.

Consequently, the optimal pH value for the determination of cellulase activity was 6.0. Similar results were found by other research: the optimal pH and temperature to achieve maximum CMCase activity of a *Bacillus amyoliquefaciens* were 7.0 and 50°C (Lee et al., 2008), of a *Clostridium thermocellum* were 5.7 to 6.1 and 70°C (Johnson et al., 1982). In these studies, the CMCase activities were close to each other with pH from 6.0 to 7.0 and temperature from 50 to 70°C, which were comparable to the results in this study. However, different optimal pH and temperature results were also found by others: Kalogeris et al. (2003) reported that the CMCase activity of a *Thermoascus aurantiacus* reached the highest at pH 3.5 and 4, and temperature 75°C; while Coral et al. (2002) reported pH 4.5 and 7.5, and temperature 40°C for obtaining maximum CMCase activity in an *Aspergillus niger.* Therefore, the current optimal pH and temperature were good for the measurement of cellulase in CSK1. Other cellulases from

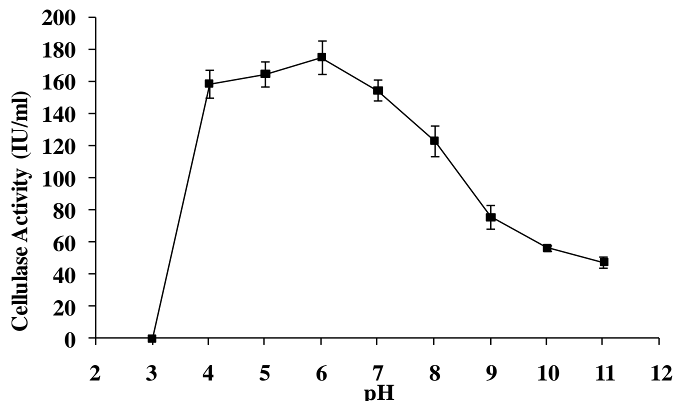

Figure 4. Cellulase activity at different reaction pH.

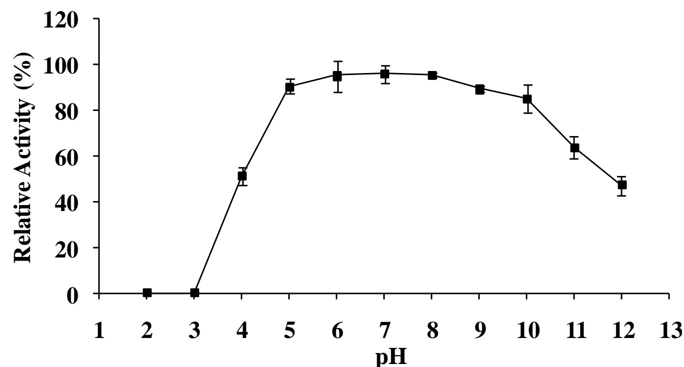

Figure 6. Cellulase stability under different pH treatments

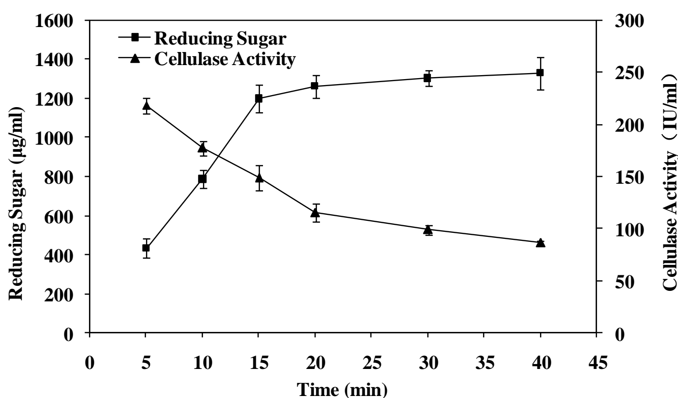

Figure 5. Cellulase activity at different reaction times.

different microbes need their own optimal pH and temperature for determining the cellulase activity.

Optimal time for the cellulase reaction

The amount of reducing sugar produced by the cellulase decomposing substrate increased from 430 to 1198 μg /mL as reaction time increased from 5 to 15 min. The change of the rate of reducing sugar slowed after 15 min, and there was no significant difference from 20 to 40 min, when the amount of reducing sugar reached around 1300 μg/mL (Figure 5). Cellulase activity on the other hand, declined gradually as time extended, and the highest value was observed when the reaction time was 5 min. However, the total amount of reducing sugar was too limited to achieve a stable result at reaction 5 min.

When the reaction time was 10 min, the amount of reducing sugar produced was in a rapid augmentation stage, and it had significantly increased as compared with the value achieved in 5 min. The cellulose activity at 15 min far exceeded that at 5 min. At a reaction time of 10 min, the cellulase activity could be more accurately provided, so the optimal time selected for the cellulase reaction was 10 min.

Stability of cellulase

The cellulase stability of CSK1 was evaluated by mixing the crude cellulase extract with different pH media, and the results indicated that there was no cellulase activity determined at pH ranging from 2.0 to 3.0, and the relative activity increased first and then decreased with the increasing pH (Figure 6). The relative CSK1 cellulase activity was higher than 90% when the pH ranged from 5 to 10, which indicated that the cellulase was relatively stable with a pH close to neutral, both too low pH (<5) and too high pH (>10) lead to a decline of the cellulase activity.

The influence of temperature on the cellulase stability was determined by measuring the relative cellulase activity of the crude cellulase extract of CSK1 that processed in 30 to 90°C for 30 min. The results indicated that the cellulase activity was basically stable and relative enzyme activity was maintained above 70% between processing temperature 30 and 70°C. However, when the processing temperature exceeded 70°C, relative enzyme activities quickly declined to 32% at 80°C, and the cellulase activity was completely lost when the temperature reached 85°C (Figure 6). Therefore, the cellulase of CSK1 could maintain a higher enzyme activity for certain times (30 min in the current study) with temperature below 70°C, which agree with its relatively high culturing temperature environment (50°C).

Different cellulases (that is, different types or from different sources) require different conditions to maintain their functional ability. Temperature and pH are the two important ambient factors that can directly influence the cellulase stability. In the production of cellulose enzyme bacteria, such as *A. niger* (Ikeda et al., 1973; Sohail et

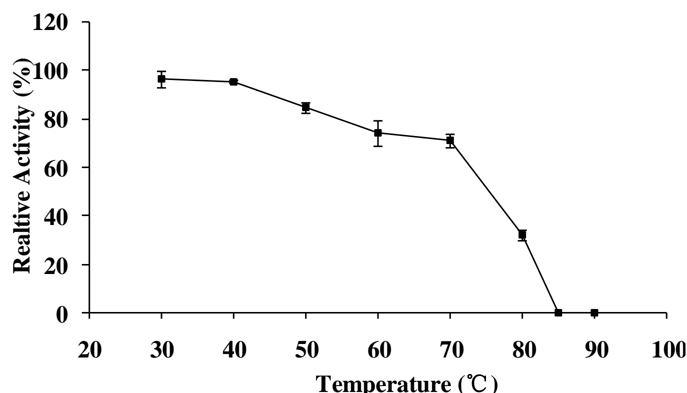

Figure 7. Cellulase stability under different temperature treatments.

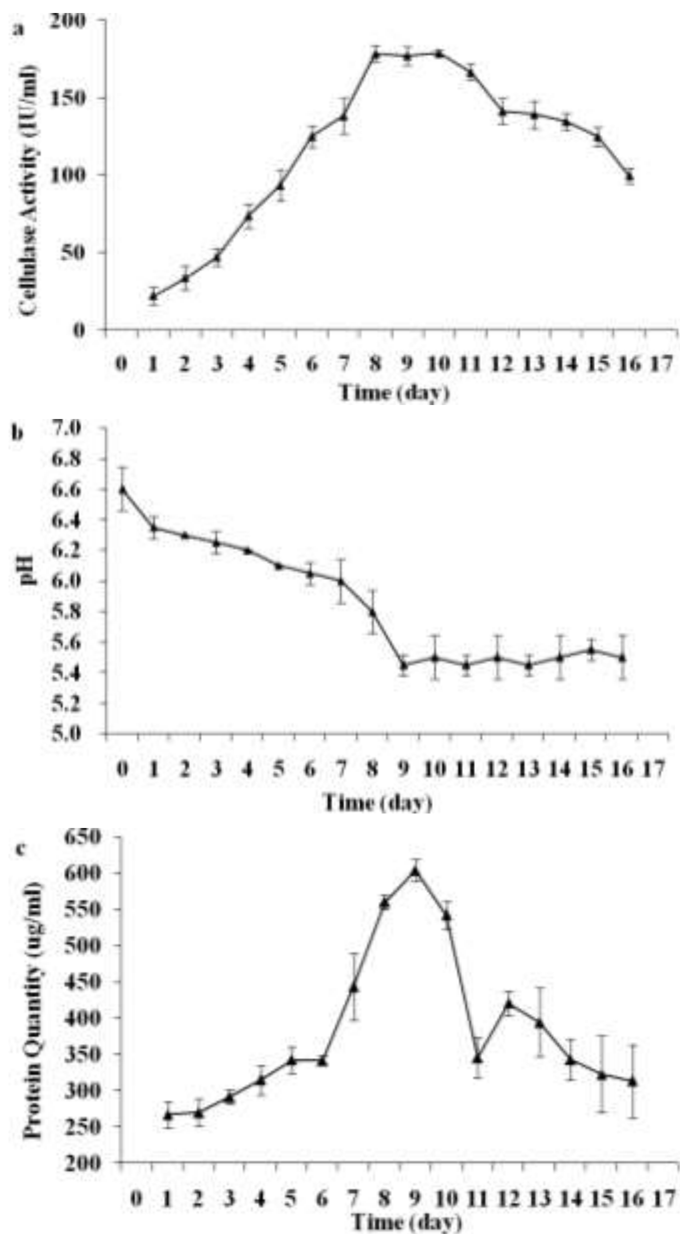

Figure 8. Cellulase activity (a), pH (b), and protein quantity (c) in the CSK1 growth cycle.

al., 2009) and *Trichoderma reesei* (Sprey and Lambert, 1983; Szengyel and Zacchi 2000), the acid enzyme is commonly produced, for which the optimal pH has typically been between 4.0 and 6.0. Some microbes also exhibit basophil and alkali resistance (Ariffin et al., 2006; Singh et al., 2004).

Therefore, the stable conditions of cellulase from different microbes might quite different. Farinas et al. (2010) studied the stability of cellulase and xylanase of *A. niger* by using response surface methodology, and reported that the optimum pH and temperature for maintaining high activities of endoglucanase, β-glucosidase and xylanase ranges from 4.0 to 5.5 and 35 to 60°C, respectively.

Lee et al. (2008) studied the influence of pH and temperature on CMCase produced by *B. amyoliquefaciens* DL-3, and found the CMCase was relatively stable ranging from pH 4.0 to 9.0 and temperature 50 to 70°C. In this study, the CSK1 cellulase activity remained high and stable below 70°C and in the pH range of 5.0 to 10.0 (Figure 7). Thus, the CMCase stable conditions of CSK1 were different to CMCase from other microorganism, also different to other types of cellulase.

Cellulase application could be more extensive if its stable ranges were larger. The large stable ranges of CSK1 CMCase were meaningful for cellulose decomposition that includes large pH changes before and after the reaction process, and in some high temperature reaction environments. These conditions are especially appropriate for composting fermentation and silage processing.

Cellulase activity, pH, and protein quantity during culture of CSK1

The cellulase activity increased with the increasing culturing time from 0 to 8 days, and reached its highest value of 182 IU/mL on day 8. Then the cellulase activity keeps high values during culturing days from 8 to 10. After day 10, the cellulase activity declined gradually with the extension of culturing time, and reached 96 IU/mL at day 16 (Figure 8a). The pH values declined gradually from 6.7 to 6.1 with culturing time from 0 to 7 days; then declined rapidly from 6.1 to 5.4 with culturing time from 7 to 9 days, and then remained nearly stable (at around 5.4) from 10 to 16 days (Figure 8b). Protein quantity also changed correspondingly, which increased gradually from 267.09 to 341.54 μg/mL with culturing time from 0 to 7 days, and then increased rapidly from 443.19-603.85 μg/mL with culturing time from 7 to 9 days. In the last 7

days (10 - 16 day), the protein quantity generally declined from 542.04 to 313.53 µg/mL (Figure 8c).

The rate of enzymatic cellulose hydrolysis was influenced by the changes of pH of the suspension, which resulted from the accumulation of acetate during cellulose degrading process. Romsaiyud et al. (2009) reported that the accumulation of acetate resulted in a decrease in pH, which resulted in the decrease of both cellulase production and cellulose hydrolysis.

In the early stage of culturing of CSK1, cellulase activity increased with the improvement of CSK1 growth, and achieved its highest value around day 8, and the protein quantity also increased to the peak correspondingly. Meanwhile, the pH of the culture solution declined gradually with the degradation of filter paper, and reach around 5.8 at day 8.

Kato et al. (2004b) reported that with the degradation of the cellulose substrate, large amounts of acid (acetic acid primarily) were produced and led to a decline of pH. In the later culturing stage, the low culture solution pH inhibited the growth CSK1, which resulted in a gradual decline in the cellulase activity and also the total amount of protein in solution.

The determination of the culturing time when the enzyme activity reaches the highest is a key procedure to evaluate the degradation efficiency of cellulase producer bacteria and cellulase itself. It took considerably longer time for CSK1 to reach the highest cellulase activity compared to MC1. This may be because the bacterial community MC1 has a vigorous metabolism, and the other bacteria of the community have a stimulative effect on CSK1 cellulase production. Thus, MC1 achieved the highest cellulase activity in a relatively short time.

Conclusion

A systematic cellulase activity determination method based on the DNS spectrophotometry was developed. Following this step, the cellulase characteristics of CSK1 were determined and evaluated. The optimal conditions for determination of CSK1 cellulase activity were obtained in terms of the measuring wavelength, the reaction temperature and pH, and the ideal reaction time. Cellulase of CSK1 was relatively stable at temperatures below 70°C, and pH between 5.0 and 10.0, which indicated high application value and development potential in practical process. Through the determination of the cellulase characteristics and activity of CSK1 in 16 days, we found significant influences with each other among cellulase activity variation, pH and CSK1 biomass changes. Corresponding adjustment measures could be applied based on such information to obtain more efficient and higher-yield processes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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REFERENCES

Anand AAP, Vennison SJ, Sankar SG, Prabhu DIG, Vasan PT, Raghuraman T, Vendan SE (2010). Isolation and characterization of bacteria from the gut of *Bombyx mori* that degrade cellulose, xylan, pectin and starch and their impact on digestion. J. Insect Sci. 10(1):107.

Aoyama A, Kurane R, Matsuura A, Nagai K (2015). Newly isolated *Penicillium oxalicum* A592-4B secretes enzymes that degrade milled rice straw with high efficiency. Biosci. Biotechnol. Biochem. 79(5):820-829.

Ariffin H, Abdullah N, Umi Kalsom MS, Shirai Y, Hassan MA (2006). Production and characterization of cellulase by *Bacillus pumilus* EB3. Int. J. Eng. Technol. 3(1):47-53.

Bailey M, Enari T, Linko M (1975). Symposium on enzymatic hydrolysis of cellulose. Aulanko, Finland, 12–14 March 1975. SITRA, Helsinki 1975. p. 525.

Bayer EA, Chanzy H, Lamed R, Shoham Y (1998). Cellulose, cellulases and cellulosomes. Curr. Opin. Struct. Biol. 8(5):548-557.

Bugg, TD, Ahmad M, Hardiman EM, Rahmanpour R (2011). Pathways for degradation of lignin in bacteria and fungi. Nat. Prod. Rep. 28(12):1883-1896.

Coral G, Arikan B, Ünaldi MN, Guvenmez H (2002). Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain. Turkish J. Biol. 26(4):209-213.

Cui Z, Li M, Piao Z, Huang Z, Ishii M, Igarashi Y (2002). Selection of a composite microbial system MC1 with efficient and stability cellulose degradation bacteria and its function. Environ. Sci. 23(3):36-39.

Cui Z, Piao Z, Wang W, Su B, Yu H (2004). Conditions of cellulase production in the presence of microbial consortia MC1 capable of degrading cellulose with high efficiency. J. Agro-environmental Sci. 23(2):296-299.

Dashtban M, Maki M, Leung KT, Mao C, Qin W (2010). Cellulase activities in biomass conversion: measurement methods and comparison. Crit. Rev. Biotechnol. 30(4):302-309.

Eveleigh DE, Mandels M, Andreotti R, Roche C (2009). Measurement of saccharifying cellulase. Biotechnol. Biofuels 2(1):21.

Farinas CS, Loyo MM, Baraldo A, Tardioli PW, Neto VB, Couri S (2010). Finding stable cellulase and xylanase: evaluation of the synergistic effect of pH and temperature. New Biotechnol. 27(6):810-815.

Ghose TK (1987). Measurement of cellulase activities. Pure Appl. Chem. 59(2):257-268.

Goksyr J, Eriksen J (1980). "Economic Microbiology, volume 5: Microbial Enzymes and Bioconversions." ed. Hose A H. London, New york, Torouto, Sydney, San francisco 5:301-306.

Guo P, Zhu W, Wang H, Lue Y, Wang X, Zheng D, Cui Z (2010). Functional characteristics and diversity of a novel lignocelluloses degrading composite microbial system with high xylanase activity. J. Microbiol. Biotechnol. 20(2):254-264.

Ikeda R, Yamamoto T, Funatsu M (1973). Purification and some physical properties of acid-cellulase from *Aspergillus niger*. Agric. Biol. Chem. 37(5):1153-1159.

Johnson EA, Sakajoh M, Halliwell G, Madia A, Demain AL (1982). Saccharification of complex cellulosic substrates by the cellulase system from *Clostridium thermocellum*. Appl. Environ. Microbiol. 43(5):1125-1132.

Kalogeris E, Christakopoulos P, Katapodis P, Alexiou A, Vlachou S, Kekos D, Macris BJ (2003). Production and characterization of cellulolytic enzymes from the thermophilic fungus *Thermoascus aurantiacus* under solid state cultivation of agricultural wastes. Process Biochem. 38(7):1099-1104.

Kato S, Haruta S, Cui ZJ, Ishii M, Yokota A, Igarashi Y (2004a). *Clostridium straminisolvens* sp. nov., a moderately thermophilic,

aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community. Int. J. Syst. Evol. Microbiol. 54(6):2043-2047.

Kato S, Haruta S, Cui ZJ, Ishii M, Igarashi Y (2004b). Effective cellulose degradation by a mixed-culture system composed of a cellulolytic *Clostridium* and aerobic non-cellulolytic bacteria. FEMS Microbiol. Ecol. 51(1):133-142.

Kato S, Haruta S, Cui Z J, Ishii M, Igarashi Y (2008). Network relationships of bacteria in a stable mixed culture. Microb. Ecol. 56(3):403-411.

Kerr RA (2007). Global warming is changing the world. Science 316(5822):188-190.

Lee YJ, Kim BK, Lee BH, Jo KI, Lee NK, Chung CH, Lee JW (2008). Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. Bioresour. Technol. 99(2):378-386.

Lewis SM, Montgomery L, Garleb KA, Berger LL, Fahey GC (1988). Effects of alkaline hydrogen peroxide treatment on *in vitro* degradation of cellulosic substrates by mixed ruminal microorganisms and Bacteroides succinogenes S85. Appl. Environ. Microbiol. 54(5):1163-1169.

Lü Y, Li N, Yuan X, Hua B, Wang J, Ishii M, Cui Z (2013). Enhancing the cellulose-degrading activity of cellulolytic bacteria CTL-6 (*Clostridium thermocellum*) by co-culture with non-cellulolytic bacteria W2-10 (*Geobacillus* sp.). Appl. Biochem. Biotechnol. 171(7):1578-1588.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31(3):426-428.

Odom JM, Wall JD (1983). Photoproduction of H_2 from cellulose by an anaerobic bacterial coculture. Appl. Environ. Microbiol. 45(4):1300-1305.

Pérez J, Munoz-Dorado J, de la Rubia TDLR, Martinez J (2002). Biodegradation and biological treatments of cellulose, hemicellulose and lignin: An overview. Int. Microbiol. 5(2):53-63.

Piao Z, Cui Z, Su B, Lu P, Wang W (2002). Characteristics of cellulase activity of a composite microbial system-MC1 with efficient and stable cellulose degradation. J. China Agric. Univ. 8(1):59-61.

Romsaiyud A, Songkasiri W, Nopharatana A, Chaiprasert P (2009). Combination effect of pH and acetate on enzymatic cellulose hydrolysis. J. Environ. Sci. 21(7):965-970.

Singh J, Batra N, Sobti RC (2004). Purification and characterisation of alkaline cellulase produced by a novel isolate, *Bacillus sphaericus JS1*. J. Ind. Microbiol. Biotechnol. 31(2):51-56.

Singh R, Kumar R, Bishnoi K, Bishnoi NR (2009). Optimization of synergistic parameters for thermostable cellulase activity of *Aspergillus heteromorphus* using response surface methodology. Biochem. Eng. J. 48(1):28-35.

Sohail M, Siddiqi R, Ahmad A, Khan SA (2009). Cellulase production from *Aspergillus niger* MS82: effect of temperature and pH. N. Biotechnol. 25(6):437-441.

Sprey B, Lambert C (1983). Titration curves of cellulases from *Trichoderma reesei*: demonstration of a cellulase-xylanase-β-glucosidase-containing complex. FEMS Microbiol Lett. 18(3):217-222.

Stellmach B (1992). Bestimmungsmethoden enzyme. Chinese Light Industry Press, Bejing China. 122-123.

Stephanopoulos G (2007). Challenges in engineering microbes for biofuels production. Science 315(5813):801-804.

Szengyel Z, Zacchi G (2000). Effect of acetic acid and furfural on cellulase production of *Trichoderma reesei* RUT C30. Appl. Biochem. Biotechnol. 89(1):31-42.

Wang W, Gao PJ (2003). Function and mechanism of a low-molecular-weight peptide produced by *Gloeophyllum trabeum* in biodegradation of cellulose. J. Biotechnol. 101(2):119-130.

Wang WD, Cui ZJ, Wang XF, Niu JL, Liu JB, Igarashi Y (2005). Capability and stability of degrading rice straw of composite microbial system MC1. Environ. Sci. 26:156-160.

Wang J, Li J, Liu J, Hua B, Wang X, Lv Y, Cui Z (2014). Influence of cell disruption and elution on cellulase release of *Clostridium straminisolvens* (CSK1). Appl. Biochem. Biotechnol. 173(2):510-521.

Wood TM, Bhat KM (1988). Methods for measuring cellulase activities. Methods Enzymol. 160:87-112.

Xiao Z, Storms R, Tsang A (2004). Microplate-based filter paper assay to measure total cellulase activity. Biotechnol. Bioeng. 88(7):832-837.

Xu G, Goodell B (2001). Mechanisms of wood degradation by brown-rot fungi: chelator-mediated cellulose degradation and binding of iron by cellulose. J. Biotechnol. 87(1):43-57.

Zhang YP, Hong J, Ye X (2009). Cellulase assays. Biofuels: Methods Protoc. 213-231.

Zhao XH, Wang W, Tong B, Zhang SP, Wei DZ (2016). A newly isolated *Penicillium oxalicum* 16 cellulase with high efficient synergism and high tolerance of monosaccharide. Appl. Biochem. Biotechnol. 178(1):173-183.

Zhe P, Zongjun C, Baolin S, Peng L, Weidong W (2003). Characteristics of cellulase activity of a composite microbial system-MC1 with efficient and stable cellulose degradation. J. China Agric. Univ. 8(1):59-61.

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Short Communication

Use of clinical clue to diagnose anaerobic oral and maxillofacial infections among patients at Muhimbili National Hospital, Dar-es-Salaam, Tanzania

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Increase in life threatening oral facial infection despite the use of antimicrobial among patients attending muhimbili national Hospital, Tanzania has made it imperative to investigate bacteria causing infections. However, comprehensive anaerobic bacteriology of clinical specimens is expensive and time consuming procedure. This study aim therefore at providing information on the use of clinical clue, to diagnose anaerobic infection among patients with oral and maxillofacial infections. A hospital based descriptive cross-sectional study was conducted on Seventy participants (age between 19 to 70yrs) among patients, attening department of oral and maxillofacial surgery of the Muhimbili national hospital in Dar es Salaam, Tanzania over a period of eight months. Study participants were interviewed using a prepared questionnaire.Special clinical form was used to check for clinical presentation of the lesion .The specimen were collected and transported in anaerobically prereduced transport medium for processing in the laboratory isolation and identification which were done employing standard bacteriologic techniques. Antibiotic sensitivity testing for isolates was, detected following the guidline of clinical and laboratory standards. 70% of patient was presented with one or more clinical sign of anaerobic infection and their entire clinical sample obtained yielded growth of anaerobes.This study revealed the need for clinicians to consider pointers of anaerobic infections, whenever clerking patients with oral and maxillofacial infections.

Key words: Anaerobes, oral and maxillofacial infection, clinical clue.

INTRODUCTION

Infections caused by anaerobic bacteria are common and may be serious and life-threatening (Manyahi et al., 2014). Oral facial infections remain a major problem in Oral and maxillofacial field in spite of the availability of potential useful antibiotics (Simon and Matee, 1999; Holmstrup et al., 2003; Lin et al., 2016).

Microbiology of oral facial infection has been widely studies and the reports shows that, various form of aerobic and anaerobic microorganism have been isolated (Simo et al., 1998; Jose et al., 2013). Treatment of oral

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facial infection as in most instances, require the use of empiric antibiotic whereby clinicians approach to the management relies on the knowledge of the likely microorganism that may cause an infection in a particular site and availability of antibiotic as per national guideline, to the rational choice of antibiotic therapy in that particular region (Ndukwe et al., 2007).

In severe forms of orofacial infections like necrotizing fasciitis, deep space infection and osteomyelitis, culture studies involving both aerobic and anaerobic bacteriology are desirable to provide information on the likely pathogenic organisms, causing disease which are likely antibiotic sensitivity (Brook, 2009; Rishi et al., 2015). Unfortunately routine clinical microbiology especially anaerobic bacteriology is expensive and requires special facilities and expertise to perform, which is not readily available in many hospitals in the developing countries even in large referral hospitals. Studies to determine presence of anaerobes and various conditions which are likely to be isolated, can be of help in providing a guide to clinician for making rational decision over the choice of antibiotic, in the management of these infections especially in areas with limited diagnostic facilities.

This study therefore aims at, providing information on the use of clinical clue to diagnose anaerobic infection among patients with oral and maxillofacial infections.

MATERIAL AND METHODS

This was a descriptive cross-sectional hospital based study, which was approved by ethic committee of Muhimbili university of Health and allied sciences. A written consent was obtained from all the patients or a legal relative of a patient.

Patient recruitment

Study was conducted in the Department of Oral and Maxillofacial Surgery of Muhimbili National hospital which is the largest referral, consulting and teaching hospital in Dar-es-salaam, Tanzania in association with Microbiology teaching Laboratory of Muhimbili University of Health and allied sciences. Seventy patients who had conditions such as Dental abscess, Infected Socket, Ludwig's angina and necrotising fasciitis were included in our study.

Interview was conducted using a structured standard questionnaire to obtain information regarding age, sex, the presenting symptoms, duration of the condition and medical history. A special clinical form was used to record the presenting clinical signs and infection characteristic of the lesion.

Bacteriological study

Pus samples were collected aseptically by aspirating the lesions, using sterile syringe during the incision and drainage or wound dressing. After aspirating, the specimen was immediately inoculated in a special anaerobic transport media (BD curl anaerobic Transport) to the laboratory within 20 min, processed for culture as early as possible within 2 h (Brook et al.,1996; Sara et al., 2015). The culture and sensitivity were conducted for the clinical specimens, obtained from the patient before initiation of any antibiotic therapy. Sample not suitable for culture such as contaminated or those that did not meet the criteria such as exposure to antibiotic was discarded.

Specimen processing and Identification

Direct smear formed a crucial role in the processing of specimen, by giving preliminary diagnosis of infection. Gram stain was done followed by examination under a microscope, using oil immersion (100 × magnification), pus cells, bacteria cells and other characteristics such as fine slender, minute, pleomorphic features which were appreciated.

Blood agar containing kanamycin and vancomycin (BD0403 CDC 5% sheep blood agar for anaerobes) was used (5µg metronidazole and 10 µg penicillin discs and 10 µg Gentamycin was placed for presumptive recognition of anaerobes and the media was incubated in anaerobic jar in atmosphere generated) using BD commercial gas generating kit in accordance with manufacturer's instructions. Plates were examined after 48 h.

Isolates were identified based on microscopic characteristics, aerotolerance test, colonial characteristic and biochemical tests (Flynn et al., 2007). Antimicrobial susceptibility pattern of isolated bacterial pathogens was conducted by agar diffusion method and E-test according to CLSI guideline for anaerobic susceptibility testing.

Statistical analysis

The statistical analysis was performed using descriptive methods. The results were expressed as per percentages for analysis of various data. Calculations were performed using SPSS 10.0. Parametric data were presented as mean +/- SD.

RESULTS

A total of 70 patients with different oral and maxillofacial infections were included in this study. Among them 41 were male (58.5%) and 29 were female (41.5%) females. Their mean age was 32 years. Thirty-seven 37(53%) of cases were dental abscesses followed by Ludwig's angina 12(17%).

Thirteen 13(19%) had necrotising fasciitis and infected socket 8(11%). Pointers of anaerobic infection noted were, foul smelling 30 (43%), necrotising gangrenous tissue 12 (17%), free gas in tissue 5 (7%) and gas discolouration exudates 8 (11%).

Disease outcome was that, 5 (7%) of the patients died in the first week of admission to hospital, after sample was collected (Table 1).

Organism isolated from different clinical conditions

Among different clinical sample processed for bacteriology, majority of obligate anaerobes were seen in conditions like Ludwig's angina 6 (42%) and Necrotising fasciitis 4 (36%). Majority of facultative anaerobes were isolated from dental abscess 23 (72%) whereas, a mixture of anaerobes and facultative anaerobes were obtained from all the conditions, except infected socket (Table 2).

Table 1. Clinical presentation of oralfacial infections.

Type of Infection	No	%
Ludwig's angina	12	17
Dental abscess	37	53
Necrotizing fasciitis	13	19
Infected socket	8	11
Pointer of anaerobic infection		
Foul smelling discharge	30	43
Necrotizing gangrenous tissue	12	17
Free gas in tissue	5	7
Black discolouration exudates	8	11
Disease outcome		
Death	5	7
Survival	65	93

Table 2. Type of organism that were isolated from different clinical conditions.

Clinical condition	No of sample	+ve obligate anaerobes	+ve facultative anaerobes	+ve obligate& facultative anaerobes
Ludwig's angina	14	6 (42%)	3 (21%)	5 (35.7%)
Dental abscess	32	2 (6%)	23 (72%)	7 (22%)
Necrotising fasciitis	11	4 (36%)	1 (9%)	6 (54%)
Infected socket	8	3 (37%)	5 (62%)	0

Table 3. Antimicrobial sensitivity patterns of bacterial isolates.

Organism Isolated	Organism isolated	Sensitive Class of antibiotic
Ludwig's angina	GPC + GNR ± anaerobes	Penicillins;cephalosporins,clindamycin,Mettronidazole
Dental abscess	GPC + GNR ± anaerobes	Penicillins;cephalosporins,clindamycin,Mettronidazole
Necrotising fasciitis	GPC + GNB obligate anaerobes	Clindamycin; metronidazole, Penicillins, carbapenem
Infected socket	Aerobic GPC GPC + GNR ± anaerobes	Penicillins; first-generation cephalosporins cephalosporin, carbapenem

Antimicrobial sensitivity patterns of bacterial isolates

Table 3 shows the antibiotic sensitivity pattern of bacterial isolates. Majority of these organisms were susceptible to β-Lactam and β-lactamase inhibitor antibiotics such as Penicillin, Clindamycin, Metronidazole, Cefalosporin and Carbapenem.

DISCUSSION

This hospital based study aimed at investigating use of anaerobic pointer, in diagnosis of oral and maxillofacial infections among patient at Muhimbili National Hospital, Dar-es-salaam, Tanzania. Bacteriological studies of etiological agents of orofacial infections especially anaerobes are very limited therefore, routinely bacteriological study to patients with severe infections is not done and hence treatment given, using broad spectrum antibiotic may not all the time delay healing or give good results. In this study, seventy percent (70%) of the patients presented with either one or more of the pointers of anaerobic infection, that is, foul smelling discharge, necrotizing gangrenous tissue, free gas in tissues and black discolouration of all their culture results showed presence of either one or more anaerobic bacteria hence this is in agreement with various study which report that, presence of clinical clue of anaerobic infections in patient although not specific when present can be suggestive of anaerobic infections (Robertson and

Smith, 2009; Akinkunmi et al., 2014).

During the study 5 (7%) died few days after admission before culture results were out, this mean that use of clinical clue to diagnose serious infection is very much recommended. Anaerobic organisms were isolated in most cases which means infection due to anaerobes have increased in comparison to past reports and therefore, this is very much important to utilise the clinical clue. In diagnose, these infections especially in areas is limited in anaerobic bacteriology practises.

Of the four main clinical conditions diagnosed, Ludwig's agina and necrotizing fasciitis were leading in the number of obligate anaerobes isolated. This could be explained by the fact that, the two conditions are at late stages of odontogenic infections and therefore, clinician should be very much considering in the combination therapy while treating such cases. Organisms causing oral facial infections are sensitive to various classes of antibiotics such as β-Lactam and β-lactamase inhibitor hence rationale use of these drugs in treating those infections can be beneficial to patients.

Conclusion

Obligate Anaerobes were isolated from patient who had clinical signs of anaerobic infections at Oral and maxillofacial department of Muhimbili National Hospital, Dar-es-Salaam, Tanzania. Use of anerobic pointers in managing orofacial infections is important especially in areas where culture and sensitivity cannot be easily done.

CONFLICT OF INTERESTS

The author(s) have not declared any conflict of interest.

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REFERENCES

Akinkunmi E, Adesunkanmi A, Lamikanra A (2014). Pattern of pathogens from surgical wound infections in a Nigerian hospital and their antimicrobial susceptibility profiles. Afr. Health Sci. 14(4):802-809.

Brook I (2009). The role of beta-lactamase-producing-bacteria in mixed infections. BMC Infect. Dis. 9:202.

Brook I, Frazier H, Gher E (1996). Microbiology of periapical abscesses and associated maxillary sinusitis. J. Periodontol. 67:608-610.

Holmstrup P, Poulsen H, Andersen L, Studodont TS, Fiehn N (2003). Oral infections and systemic diseases. Dent. Clin. North Am. 47(3):575-598.

Jose FS, Isabela NR (2013). Microbiology and Treatment of Acute Apical Abscesses. Clin. Microbiol. Rev. 26(2):255-273.

Lin Y, Yan-bo L, Ao-lei G, Hai-Yan F (2016). Microbiological examination to investigate the differences in microorganisms and antibiotic sensitivity of head and neck space infections. Biomed. Res. 27(2):976-1683.

Manyahi J, Matee M, Majigo M, Moyo S, Mshana S, Lyamuya E (2014). Predominance of multi-drug resistant bacterial pathogens causing surgical site infections in Muhimbili National Hospital, Tanzania. BMC Res. Notes 7:500.

Ndukwe C, Ugboko I, Akinwande A, Ogundipe K, Esan A, Ohadugha O, Adekoya- Sofowora C (2007). Bacterial infections of the head and neck fascial spaces in Nigerians. West Afr. J. Med. 26:126-30

Rishi KB, Parveen S, Shivani G, Avneet K, Priya G (2015). A review of complications of odontogenic infections. Natl. J. Maxillofac. Surg. 6(2):136-143.

Robertson D, Smith A (2009). The microbiology of the acute dental abscess. J. Med. Microbiol. 58:155-162.

Sara Z, Nihel KK, Ilhem B, Amina H, Nadia A, Fatima N, Lotfi G (2015). Oral microflora of supragingival and subgingival biofilms in Algerian healthy adults. Afr. J. Microbiol. Res. 9(23):1548-1557.

Simo R, Hartley C, Rapado F, Zarod P, Sanyal (1998). Microbiology and antibiotic treatment of head and neck absences in children. Clin. Otolaryngol. 23:164-168

Simon E, Matee M (1999). Cervico-facial necrotising fasciitis occurring with facial paralysis: Case report. East Afr. Med. J. 76:472-474.

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