Identification Profile of *Micrococcus luteus* Cs Associated with Fermented Corn-Soybean Wastes-Meal

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Abstract: *Micrococcus* spp were among the predominant organisms isolated from earlier developed fermented corn-soybean wastes meal meant for human consumption. Hence the study was aimed at identifying the Micrococcus isolates to their species level. The *Micrococcus* spp were first subjected to phenotypic analysis and thereafter followed by genotypic analysis using 16SrRNA sequencing method after their DNA isolation and polymerization processes. The phenotypic and genotypic analyses confirmed all the *Micrococcus* isolates to be *Micrococcus luteus* Cs which had 99% relatedness to *Micrococcus luteus* NCTC 2665. The identification of the *Micrococcus* spp as *Micrococcus luteus* Cs suggested that the developed meal can be utilized for human consumption since strains of *M. luteus* are generally regarded as harmless bacteria.

Key words: Corn-soybean wastes -meal, Identification, *Micrococcus luteus*$C_{s,}$ Phenotypic, Genotypic

INTRODUCTION

Micrococci are Gram-positive cocci of 0.5 to 3.5 micrometers in diameter and are arranged in tetrads or irregular clusters. They are generally characterized by their ability to aerobically produce acid from glucose and hydrolyze aesulin (Fox *et al*., 2010).Some *Micrococcus* spp are pigmented as seen in *M. luteus* that produces yellow colonies. Majority are oxidase-positive, and this can be used to distinguish them from other bacteria such as *Staphylococcus* spp, which are generally oxidase-negative.

M. luteus has one of the smallest genomes of all bacterial cells. Hybridization studies show no close genetic relationship among the species of Micrococcus. For example, *M. luteus* and *M. lylae* are 40-50% genetically different. *M. luteus* has a G-C content of 65-75 mol% while *M. varians* has a G-C content of 66-72mol% (Pawar *et al*., 2016). About half of the strains of *M. luteus* were found to carry plasmids 1 to 100MDa in size and their genome encodes only four sigma factors and 14 response regulators, an indication of the adaptation to a strict ecological niche including mammalian skin (Young *et al.,* 2009*)*. These characteristics of *M. luteus* make them suitable for use in various applications, including bioremediation, biodegradation, wastewater treatment, drain cleaning and degreasing (Liu *et al.*, 2000). They are growth promoters of plants and fish and also involved in the production of enzymes and antibiotics (Akbar *et al.,* 2014).*Micrococcus luteus* is generally considered to be non-pathogenic and is rarely isolated from damaged tissues (Kocur *et al.,*2006).It has been reported to have produced antimicrobial metabolites and exhibited good probiotics properties (Greenblatt *et al.,* 2004; Ganz *et al.,* 2002). In addition, *Micrococcus luteus,* which was isolated from gonads and intestines of apparently healthy *Oreochromis niloticus* was found to be safe for *O. niloticus* and had antagonistic effect against the pathogenic *Aeromonas hydrophila* (the cause of Aeromonas septicemia among fresh water fish) (Greenblatt *et al.,*2004; Akbar *et al.,* 2014). The *M. luteus* has been isolated from human skin where it helped to break down components in the sweat into compounds associated with bad odor. They can also be isolated from water, dust, soil, dairy products such as milk and fermented products such as beer (Kocur *et al.,* 2006). Since strains of *Micrococcus luteus* which are beneficial to man have been associated with some fermented products, the study was focused on full identification of *Micrococcus* spp predominant in the developed fermented corn-soybean waste meal.

MATERIALS AND METHODS

Bacteria Recovery from the Developed Fermented Meal

The production process of the fermented corn-soybean meal has been earlier described (George-Okafor *et al.,* 2018).The fermented waste meal (1% w/v) was serially diluted with sterile water and 0.1ml of the diluted samples were inoculated unto various media including Nutrient agar and Mannitol salt agar (Oxoid) and incubated for 24h at 37^{o}C. The developed colonies were subjected to identification after obtaining their pure cultures.

Examination of the Pure Cultures

The macroscopic and microscopic examinations were done as described by Fazlani *et al.* (2008).The colour and sizes of colonies on the agar plates were observed. The colonies that had yellow pigmentation were Gram stained for microscopic characterization. Only the Gram positive and cocci that were in clusters were subjected to biochemical tests.

Biochemical Tests on the Cocci Isolates

The Gram+ve cocci were biochemically examined following tests on catalase, oxidase, urease, coagulase, nitrate reduction, motility, bacitracin-sensitivity and production of acid from carbohydrates such as glucose, sucrose, lactose, fructose, galactose and maltose (Liu *et al.,*2000).

Isolation of DNA Molecules from the Isolates

The cocci cells (2%) were fully activated in Nutrient broth to obtain 24h culture for the extraction of their DNA molecules. The method as described by George-Okafor *et al.* (2018) was applied using Zymo reagents.

DNA Sequencing by 16S rRNA Method

The 16SrRNA gene sequence analysis was performed as described by Zhang *et al.*(2000), at Humanizing Genomics Macrogen Inc. 1001 World Meridian Center, 60-24 Kasan-dong, Kumchun-Ku Seoul, Korea. Each PCR contained 20ng DNA in a 30ml reaction mixture of EF- Taq (SolGent, Korea) and 10mm of each primer notably 785F (GGA TTA GAT ACC CTG GTA), 27F (AGA GTT TGA TCM TGG CTC AG), 907R (CCG TCA ATT CMT TTR AGT TT) and 1492R (TAC GGY TAC CTT GTT ACG ACT T) .Cycling conditions were set at an initial denaturation at 95^{o}C for 3min, followed by 35cycles of 95^{o}C for 1min, annealing at 55^{o}C for 1min, elongation at 72^{o}C for 1min and finishing with at 72^{o}C for 10min. The amplification products were purified with a multi-screen filter plate (Millipore corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM Big Dye Terminator V3.1 cycle sequencing kit. The DNA samples containing the extension products were added to Hi – Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95^{0}C for 5min, followed by 5min on ice and then analyzed by ABI prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Characterization of Cocci Isolates

Identification through morphological and biochemical tests as stated in table 1 revealed that Micrococcus isolates were catalase, oxidase, urease positive and coagulase negative. They were bacitracin sensitive, motility negative and had positive reaction to nitrates. However, they were not able to ferment glucose, sucrose, lactose, fructose, galactose and maltose.

These observed characteristics are in line with the characteristics associated with *Micrococcus* spp as reported by Kocur *et al.* (2006); Fox *et a*l. (2010) and Pereira *et al.* (*2012*). Their inability to grow on MSA and showing negative oxidase test clearly distinguished the isolates from *Staphylococcus aureus* which can cause food intoxication. Table 2 shows partial genome sequencing of *Micrococcus luteus* C_s. The genotypic characterization revealed that *Micrococccus luteus* Cs had partial sequence length of 1525, Score of 2639 bits with identities of 1453/1464, indicating 99% identity to *Micrococccus luteus* strain NCTC 2665.

These characteristics gave a clearer picture for the confirmation of the isolates as *M. luteus* Cs. Although the *Micrococcus* spp were recovered from different agar plates, yet all had the same genotypic characteristics (table 3). It is an indication that they were predominant sp in the developed meal.

The applied 16SrRNA molecular analysis has been used by many authors in the identification of various *Micrococcus* spp from various sources (Pawar *et al.,* 2016; Young *et al.*, 2009).This is because the genotypic analysis helps to identify the bacteria down to species level (Mohagnia *et al.* 2008). The observed short genomic length of 1525 is in accordance with the findings of Haga *et al.* (2003) which stated that *M. luteus* has one of the smallest genomes among the bacterial cells and its hybridization studies show no close genetic relationship among other species of Micrococcus. The recovery of *Micrococcus luteus*Cs from the fermented waste meal is interesting as it promotes the possible utilization of the meal for human consumption. This is because of the reported probiotic potential of *M. luteus* (Ganz *et al.,* 2002; Greenblatt *et al.,* 2004; Akbar *et al.,* 2014).

Table 1: Phenotypic characteristics of Cocci Isolates

BCI	Colonial Appearance on		GR	Ca	Ox	Nitr	Mt	Bct	Ur	Co	Sugar Fermentation						Suspected Organisms
											G	S	L	F	Ga	Ma	
	NA	MSA															
IS_1	Bright yellow colonies	-	+cocci in irregular clusters	+	+	+	-	S	+	-	-	-	-	-	-	-	*Micrococcus* sp1
IS_2	Bright yellow colonies	-	+cocci in tetrads/ irregular clusters	+	+	+	-	S	+	-	-	-	-	-	-	-	*Micrococcus* sp2
IS_3	Bright yellow colonies	-	+cocci in tetrads	+	+	+	-	S	+	-	-	-	-	-	-	-	*Micrococcus* sp3
IS_4	Bright yellow colonies	-	+cocci in tetrads	+	+	+	-	S	+	-	-	-	-	-	-	-	*Micrococcus* sp4

Legend: BCI-Bacterial cocci isolates; NA- Nutrient Agar; MSA- Mannitol Salt Agar; S-Sensitive, IS-Isolates; sp-species, GR- Gram reaction, Ca- Catalase, Ox- Oxidase, Nitr-Nitrate reduction, Mt- Motility, Bts- Bacitracin-sensitivity, Ur- Urease, Co- Coagulase, G-Glucose, S- Sucrose, L- Lactose, F- Fructose, Ga- Galactose, Ma- Mannitol.

Table2: Partial Genome Sequencing of *Micrococcus luteus* Cs with *Micrococcus luteus* Strain NCTC 2665 as the Subject Strain

Query 1188
AGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTTCTCGA
1247|||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct 420849
AGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTTCCCGA 420908
Query 1248
TCGCCGTAGAGATACGGTTTCCCCTTTGGGGCGGGTTCACAGGTGGTGCATGGTTGTCGT 1307
|||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct 420909
TCGCCGTAGAGATACGATTTCCCCTTTGGGGCGGGTTCACAGGTGGTGCATGGTTGTCGT 420968

Query 1308
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGT 1367
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 420969
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGT 421028

Query 1368
TGCCAGCACGTCGTGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG 1427
|||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421029
TGCCAGCACGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTG 421088

Query 1428
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCC 1487
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421089
AGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCC 421148
Query 1488
GGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCG 1547
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421149
GGTACAATGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCG 421208
Query 1548
GATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAA 1607
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 421209
GATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAA 421268
Query 1608
CGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGG 1667
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct 421269
CGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGG 421328
Query 1668 TAACACCCGAAGCCGGTGGCCTAACCCTTGTGGG-
GGGAGCCGTCGAAGGTGGGACCGGC 1726
|||||||||||||||||||||||||||||||| |||||||||||||||||| ||
Sbjct 421329
TAACACCCGAAGCCGGTGGCCTAACCCTTGTGGGAGGGAGCCGTCGAAGGTGGGACCAGC 421388
Query 1727 GATTGGGACTAA-TC-TAA-AAGG 1747
|||||||||||| || ||| ||||
Sbjct 421389 GATTGGGACTAAGTCGTAACAAGG 421412

Table3: Molecular Characterization of the *Micrococcus* sp

IS	Subject				Score					Identities			
	Accession no	Length	Start	End	CV	Bit	EV	Match	Pct (%)	Kd	Fm	G	Sp
MS1	NR-075062.1	1525	23	1486	96	2639	0.0	1453/1464	99	Bacteria	Micro-coccaceae	*Micro-coccus*	*M. luteus*
MS2	NR-075062.2	1525	23	1486	96	2639	0.0	1453/1464	99	Bacteria	Micro-coccaceae	*Micro-coccus*	*M. luteus*
MS3	NR-075062.3	1525	23	1486	96	2639	0.0	1453/1464	99	Bacteria	Micro-coccaceae	*Micro-coccus*	*M. luteus*
MS4	NR-075062.4	1525	23	1486	96	2639	0.0	1453/1464	99	Bacteria	Micro-coccaceae	*Micro-coccus*	*M. luteus*

Key: IS- Isolates, MS- *Micrococcus*spp, CV- Coverage, EV- Expected value, Kd- Kingdom, Fm- Family, G- Genus, Sp- Species

CONCLUSION

The results of the study indicated that *Micrococcus luteus*Cs was the predominant *Micrococcus* spp domiciled in the fermented corn-soybean meal. Their presence in the meal cannot be associated with health problems as they are generally regarded as useful and harmless organisms.

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