Nutrient effects on ten *Streptomyces* spp. sporulation

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The effects of nutrients on sporulation of ten *Streptomyces* spp. was studied on 26 media. The media included starch-casein-KNO_3, glycerol-arginine (GA), actinomycete-isolation (AI), tryptone-yeast extract-glucose (TYG), and tryptone-soy agars. Media compositions had complicated effects on sporulation. The sporulation on media with minerals of GA agar and yeast extract was usually better than on media with minerals of AI agar. Glycerol often decreased sporulation, although GA agar was among the best media for sporulation. The average sporulation on media with Na-caseinate and asparagine was better than on same media with NH_4NO_3, however, a medium with Na-propionate, NH_4NO_3 and yeast extract was among the best for sporulation. At pH 5.5 on TYG-agar with minerals of GA agar instead of yeast extract all strains completely covered mycelium with spores. The sporulation capacity of strains was excellent, the mean mycelium area covered with spores on 26 media being 78 % in fourteen days. When environmental streptomycetes are able to germinate, grow, and then sporulate, they likely form a great amount of spores independent of growth conditions.

Keywords: Streptomycetes; sporulation; nutrients.

1. Introduction

The filamentous gram-positive bacteria of genus *Streptomyces* undergo a complex cycle of morphological differentiation. The germinating spores form a branched, vegetative mycelial mass, which produces aerial mycelia and subsequently spore chains [1, 2]. The cascade of events related to these morphological and physiological alterations is apparently dependent on both species-specific properties and environmental factors, such as nutrients. The exhaustion of nutrients, including nitrogen and phosphorus, and starvation media have been among conditions inducing sporulation. On the other hand, complex organic media containing yeast extract, oatmeal extract etc. have also been reported to ensure abundant sporulation. Other environmental stimuli enhancing sporulation have been mineral rather than organic sources of nitrogen, increased concentration of ions or agar, several amino acids like methionine, humic and fulvic acids, biotin, and acid or alkaline conditions [3-13]. To compare these contradictory reports, we present sporulation results on 26 different media of ten *Streptomyces* spp. Isolated from the indoor environment. The mycelium biosynthesis on same media had a tendency to increase in the order: starch – casein < glycerol – arginine < glucose – tryptone, and NH_4NO_3 < Na-caseinate – asparagines [14].

2. Materials and methods

Ten *Streptomyces* strains VTT E-99-1326 to VTT E-99-1335 (VTT; Technical Research Centre of Finland, Biotechnology and Food Research) used in the study were isolated from buildings, and characterised as presented [15-17]. The 26 media were starch-casein-KNO_3 agar (medium 6), actinomycete-isolation agar with 0.01 g/l of $FeSO_4$ x 7 H_2O instead of 0.001 g/l (medium 16) [10], glycerol-arginine agar (medium 21) [18], tryptone-yeast extract-glucose (TYG) agar (medium 23),

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tryptone-soy (TS) agar (medium 26) (Unipath Ltd., Hampshire, UK), and 21 combinations of these media (Table 1). The media were solidified with 1.5 % agar, and the pH 7.0-7.2 was adjusted with 1 M NaOH or HCl before the autoclaving at 121 °C for 15 min. *Streptomyces* spp. were grown on TYG agar until sporulation. The plates were flooded with sterile, distilled water, and the resulting spore suspension was harvested [19]. The spore suspension of 5 µl containing >5 x 10^7 spores ml^{-1}, determined by acridine orange staining and counting [20], was inoculated in triplicate on media in petri dishes 90 mm in diameter. The plates were incubated at the temperature of 20 ± 2 °C, and sporulation results were recorded after 14-16 days by the visual assessment to form five groups presented as 20, 40, 60, 80 and 100 % of mycelium covered with spores. Microscopy (Labophot-2, Nikon, Tokyo, Japan) was used to confirm sporulation. To estimate error, mean of standard deviations (SDs) of 10 strains sporulation in two independent experiments for 14-16 days was calculated. The mean of SDs on media 6, 11, 20, 21, 23, 24, and 26 were 17.0 % (range of variation in SDs, 0-30.0 %), 3.0 % (0-10.0 %), 9.0 % (0-30.0 %), 2.0 % (0-10.0 %), 4.0 % (0-10.0 %), 2.0 % (0-20.0 %), and 3.0 % (0-10.0 %), respectively. Principal component analysis (PCA) of sporulation results was performed using SPSS (SPSS/PC+ Inc, Chicago, USA). The chemicals were from Merck (Darmstadt, Germany) with following exceptions: Na-propionate, L-arginine and L-asparagine monohydrate, Fluka Chemie (Buchs, Switzerland); casein and Bacto-agar, Difco Laboratories (Detroit, MI); Na-caseinate and $Fe_2(SO_4)_3$ x $6H_2O$, Sigma (St. Louis, MO.); tryptone and yeast extract, Unipath (Hampshire, UK); glycerol, BDH Laboratory Supplies (Poole, UK); KNO_3, J.T. Baker B.V. (Deventer, Netherlands).

3. Results and discussion

On media 1-6, the *Streptomyces* spp. sporulation was the poorest on starch-casein-KNO_3 agar (medium 6), altering between 20-80 % so that 5 strains had 80 % of mycelium covered with spores (Tables 1 and 2). When minerals of starch-casein-KNO_3 agar (medium 6) were replaced with those of glycerol-arginine agar (medium 5) sporulation of six strains increased, while the replacement with yeast extract (medium 4) increased sporulation of six strains and decreased that of two strains. The removal of KNO_3 from yeast extract medium 4 and starch-casein-KNO_3 agar (medium 6) increased sporulation of three strains in resulting media 1 and 3, respectively, with concomitant decrease in sporulation of two strains on medium 3. The removal of KNO_3 from medium 5 with minerals of glycerol-arginine agar decreased sporulation of five strains (medium 2). On medium 7 without trace elements sporulation of four strains was better than on starch-casein-KNO_3 agar (medium 6). Altogether, on media from this group the greatest area of mycelium was covered with spores on medium 5 with minerals of glycerol-arginine agar and KNO_3 (8 strains 80-100 %), and on yeast extract media 1 (9 strains 80-100 %) and 4 (7 strains 80-100 %).

On media 8-19, sporulation was among the poorest on actinomycete-isolation agar (medium 16), and on glycerol media 17-19 with NH_4NO_3 as a nitrogen source (Tables 1 and 2). The removal of glycerol from media 14-19 typically increased or did not affect sporulation on respective media 8-13 independent of the other media components. However, sporulation on media 9, 12, 15 and 18 containing minerals of glycerol-arginine agar was usually better than, or equal to that on respective media 10, 13, 16 and 19 with minerals of actinomycete-isolation agar. The change from Na-caseinate and asparagine (media 8-10 and 14-16) to NH_4NO_3 (media 11-13 and 17-19) decreased or did not affect sporulation more often than increased. In exception, medium 11 containing NH_4NO_3, Na-propionate and yeast extract supported one of the best sporulation of 80-100 %. The media containing Na-caseinate, asparagine and Na-propionate without glycerol, that is media 8 (60-100 %), 9 (80-100 %) and 10 (80-100 %) were also among the best for sporulation. The best glycerol medium for sporulation of 40-100 % was medium 15 with Na-propionate, glycerol, Na-caseinate, asparagine and minerals of glycerol-arginine agar.

On media 20-22, sporulation was high between of 80-100 % on glycerol-arginine agar (medium 21), and was not affected when minerals of glycerol-arginine agar were changed to yeast extract (medium 20) (Tables 1 and 2). However, the change to minerals of actinomycete-isolation agar (medium 22) decreased the spore formation.

Table 1. The media compositions (medium 6, starch-casein-KNO_3 agar; medium 16, actinomycete-isolation agar; medium 21, glycerol-arginine agar; medium 23, tryptone-yeast extract-glucose agar; medium 26, tryptone-soy agar).

Compound	Medium number																									
(g l^{-1})	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Starch	10.0	10.0	10.0	10.0	10.0	10.0	10.0																			
Glucose																							1.0	1.0	1.0	
Glycerol														5.0	5.0	5.0	5.0	5.0	5.0	12.5	12.5	12.5				
Na-propionate								4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0							
Soy																										5.0
Casein	0.3	0.3	0.3	0.3	0.3	0.3	1.0																			
Na-caseinate								2.0	2.0	2.0				2.0	2.0	2.0										
Tryptone																							5.0	5.0	5.0	15.0
Asparagine								0.1	0.1	0.1				0.1	0.1	0.1										
Arginine																										
KNO_3				2.0	2.0	2.0																				
NH_4NO_3											4.0	4.0	4.0				4.0	4.0	4.0							
Yeast extract	2.5			2.5				2.5			2.5			2.5			2.5			2.5			2.5			
NaCl	2.0	2.0	2.0	2.0	2.0	2.0														1.0	1.0	1.0				5.0
K_2HPO_4		1.0	2.0		1.0	2.0	0.5		1.0	0.5		1.0	0.5		1.0	0.5		1.0	0.5		1.0	0.5		1.0	0.5	
$CaCO_3$			0.02			0.02																				
$MgSO_4 \times 7\ H_2O$		0.50	0.05		0.50	0.05			0.50	0.10		0.50	0.10		0.50	0.10		0.50	0.10		0.50	0.10		0.50	0.10	
$Fe_2SO_4 \times 7\ H_2O$			0.01			0.01				0.01			0.01			0.01			0.01			0.01			0.01	
$Fe_2(SO_4)_3 \times 6\ H_2O$		0.01			0.01				0.01			0.01			0.01			0.01			0.01			0.01		
$CuSO_4 \times 5H_2O$		0.001			0.001				0.001			0.001			0.001			0.001			0.001			0.001		
$ZnSO_4 \times 7H_2O$		0.001			0.001				0.001			0.001			0.001			0.001			0.001			0.001		
$MnSO_4 \times H_2O$		0.001			0.001				0.001			0.001			0.001			0.001			0.001			0.001		

Table 2. Ten *Streptomyces* spp. sporulation on 26 media fourteen days after the inoculation, evaluated as 0, 20, 40, 60, 80 and 100 % of mycelium covered with spores (medium 6, starch-casein-KNO_3 agar; medium 16, actinomycete-isolation agar; medium 21, glycerol-arginine agar; medium 23, tryptone-yeast extract-glucose agar; medium 26, tryptone-soy agar).

Strains	Medium number																										Average
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	(%±SD)
E-99-A1326	80	100	80	60	100	60	60	100	100	80	100	100	80	80	80	20	100	0	20	80	100	40	100	100	100	100	78 ± 29
E-99-A1327	60	100	60	40	100	80	100	80	100	80	100	100	80	60	60	40	60	40	40	100	100	100	100	80	60	100	78 ± 23
E-99-A1328	100	100	80	100	100	80	100	100	100	100	100	100	80	100	100	100	0	80	60	100	100	80	100	100	100	80	90 ± 21
E-99-A1329	80	80	80	80	80	80	80	60	100	80	100	80	80	80	100	60	100	100	80	60	80	60	40	40	40	80	76 ± 18
E-99-A1330	80	80	80	60	80	80	80	60	100	100	100	80	80	60	100	60	60	80	60	80	80	80	60	100	60	100	78 ± 15
E-99-A1331	100	80	80	100	100	20	80	80	80	80	80	100	60	40	100	60	20	20	20	100	100	40	40	100	80	40	69 ± 30
E-99-A1332	100	40	60	100	60	60	60	100	100	100	100	100	100	80	100	100	100	100	100	100	100	100	100	100	100	100	91 ± 18
E-99-A1333	100	60	60	100	100	40	80	100	100	100	100	60	60	80	40	0	20	100	60	100	80	80	100	100	100	40	75 ± 29
E-99-A1334	100	60	40	100	100	80	80	100	80	80	100	20	20	100	80	60	0	80	40	100	80	60	100	100	80	20	72 ± 31
E-99-A1335	80	20	40	80	40	40	40	100	100	100	80	20	80	100	100	80	80	0	0	80	100	60	100	100	100	100	70 ± 34
Average (%±SD)	88±14	72±27	66±16	82±22	86±21	62±22	76±18	88±17	96±8	90±11	96±8	76±32	72±21	78±20	86±21	58±32	54±41	60±41	48±30	90±14	92±10	70±22	84±26	92±19	82±22	76±31	78 ± 26

On media 23-26, seven strains completely covered mycelium with spores on TYG-agar (medium 23) (Tables 1 and 2). When yeast extract of TYG-agar was changed to minerals of glycerol-arginine agar (medium 24), sporulation of eight strains was 100 %, whereas after change to mineral of actinomycete-isolation agar (medium 25), five strains covered mycelium with spores. On TS-agar (medium 26), five strains covered mycelium with spores, while sporulation or three strains was 20-40 %.

The PCA of sporulation results separated 26 media according to their ability to support sporulation along the PC1 axis, explaining 33.4 % of variance in data (Fig. 1a; Tables 1 and 2). The best media for sporulation with positive scores were positioned to the right, and the poorest to the left along the PC1 axis. Sporulation on media with minerals of glycerol-arginine agar (media 2, 5, 9, 12, 15, 18, 21, 24) and yeast extract (media 1, 4, 8, 11, 14, 17, 20, 23) was usually better than on media with minerals of actinomycete-isolation agar (media 3, 6, 10, 13, 16, 19, 22, 25, respectively). Thus, Cu, Mn and Zn in minerals of glycerol-arginine agar may have favoured sporulation of *Streptomyces* spp. On the other hand, the increased ion concentration has earlier been related with the enhancement of sporulation [6] and, thus, it is actually not known whether the effect resulted from specific ions or from the increased ion concentration. On media containing minerals of actinomycete-isolation agar sporulation was high only in the presence of nutrients, like propionate, Na-caseinate and asparagine (medium 10), or glucose and tryptone (medium 25). Interestingly, complex nutrients, like yeast extract, have also been reported to support sporulation. In submerged culture, sporulation has been best induced by a nutritional downshift from a rich medium [4, 6, 12, 21, 22].

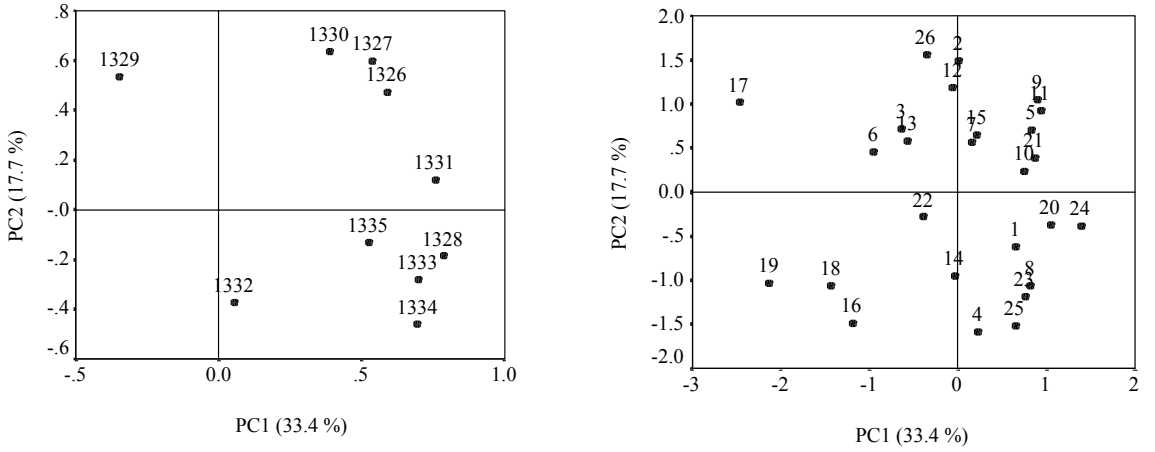

Figure 1. PCA score (a) and loading (b) plots of *Streptomyces* spp. sporulation results on 26 media used in the experiments (medium 6, starch-casein-KNO_3 agar; medium 16, actinomycete-isolation agar; medium 21, glycerol-arginine agar; medium 23, tryptone-yeast extract-glucose agar; medium 26, tryptone-soy agar).

The supplementation of glycerol to the media 8-13 often decreased sporulation in resulting media 14-19, although glycerol-arginine agar (medium 21) was one of the best media for sporulation (Tables 1 and 2). In the presence of minerals of glycerol-arginine agar, glucose and tryptone (medium 24) had a tendency to support the better sporulation than starch and casein (media 2 and 5). When minerals of glycerol-arginine agar were changed to yeast extract, the average sporulation on medium 23 with glucose and tryptone was nearly equal to that on media 1 and 4 with starch and casein. The removal of KNO_3 from medium 5 with starch and casein decreased sporulation on resulting medium 2. The change from Na-caseinate and asparagine to NH_4NO_3 (media 8-10 and 14-16) decreased the average sporulation, with the exception of yeast extract medium 11 supporting the best sporulation of all media. Thus, organic nitrogen source could support the better sporulation than mineral, opposite to the earlier reports in literature [3, 5, 6]. In submerged cultures containing peptone no sporulation occurred [23,24].

Altogether, most of the components of growth media reported earlier to support sporulation did that also in this study, such as complex organic media and mineral nitrogen sources [5, 6]. However, the results also demonstrated that these demands for sporulation can not be generalised. Nutrients had rather variable effects on sporulation of *Streptomyces* spp. The differences in nutrient concentrations affect their ratios, such as the ratio of C/N and C/P, and consequently on the formation of growth restricting conditions, like nitrogen or phosphate starvation triggering the sporulation [1, 2]. In addition, differences in nutrients may also affect the mode of cellular metabolism, such as starch and glucose-ammonium cultures support balanced glycolytic and respiratory tricarboxylic acid cycle activities without acid production. In contrast, glucose combined with minerals or a single amino acid as a nitrogen source favour fermentation and organic acid production [16, 25-28]. The heterogeneity of the system containing mixture of mature and dormant spores, spores under maturation, vegetative and aerial mycelia and reproductive hyphae may also affect both the nutrient demand and sporulation rate. Consequently, the need for specific nutrients of even one microorganism may greatly vary depending on the overall growth conditions. In this study, the best media for sporulation contained minerals of glycerol-arginine agar (media 5, 9, 15, 21 and 24), or yeast extract (media 1, 4, 8, 11, 20 and 23) (Tables 1 and 2). In addition, media 10 and 25 with minerals of actinomycete-isolation agar were among the best for sporulation. The best media for growth have been those containing yeast extract (media 1, 4, 8, 11, 14, 17, 20 and 23), glucose (media 23-25), or tryptone (23-26) [14]. When growth and sporulation results are evaluated together, *Streptomyces* spp. are likely to sporulate well after the greatest mycelium biosynthesis on media 1, 4, 8, 11, 20, 23, 24 and 25. Finally, the change in pH from 7.0 to 5.5 on medium 24 having yeast extract of TYG-agar replaced with minerals of glycerol-arginine agar has further been shown to increase sporulation of all strains to 100 % [9]. Similarly, streptomycetes have preferentially been reported to germinate and grow under the organic load often with slightly acidic pH in living environment [10].
The PCA of sporulation results separated 26 media along the PC2 axis according to the strain-specific differences in sporulation, explaining 17.7 % of variance in the data (Fig. 1b; Tables 1 and 2). For example, *Streptomyces* strain 1329 sporulated well, and strains 1332 and 1335 poorly on media 2, 3, 5, 6 and 7, whereas opposite results were obtained on media 8, 14, 16, 23, 24 and 25. Sporulation efficiency of strains also varied. The average mycelium area covered with spores on 26 media was as high as 90±21 and 91±18 % for strains 1328 and 1332, respectively, whereas the average sporulation was only 69±30, 72±31 and 70±34 % for strains 1331, 1334 and 1335, respectively (Table 2). The average sporulation of ten strains on 26 media was 78±26 % of mycelium covered with spores in 14 days. Thus, *Streptomyces* spp. sporulated quite well on all media. The strains belong to two to three branches of streptomycetes most common environmental isolates. The 16S rDNA sequence similarity of over 99.7 % was found between strains 1329 and 1330, and *Streptomyces albidoflavus* DSM 40455^T, strain 1333 and *Streptomyces coelicolor* A3(2), and the rest of strains and *Streptomyces griseus* ATCC 10137 [17]. Thus, once streptomycetes isolated from the indoor environment are able to germinate, grow, and further sporulate, they are likely to form a great amount of spores independent of growth conditions.

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